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**Phenotypic and Target-based Strategies  
for Drug Discovery in Trypanosomes**

Tese do 3º Ciclo de Estudos Conducente ao  
Grau de Doutoramento em Ciências Farmacêuticas -  
Microbiologia

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Fevereiro, 2016



DE ACORDO COM A LEGISLAÇÃO EM VIGOR, NÃO É PERMITIDA A REPRODUÇÃO  
DE QUALQUER PARTE DESTA TESE.





This work was performed at the Parasite Disease Group of the Institute for Molecular and Cell Biology (IBMC, Porto, Portugal) now part of Institute of Investigation and Innovation in Health (I<sub>3</sub>S, Porto, Portugal) in collaboration with the Center for Neglected Diseases Drug Discovery of Institut Pasteur Korea (CND3, Bundang, South Korea), now located at Laboratório Nacional de Biociências (LNBio, Campinas, Brazil). The author has received financial support through an individual PhD Studentship from Foundation for Science and Technology (FCT, Portugal) with the reference SFRH/BD/81604/2011. The experimental research performed in this work has received financial support from FEDER funds through the Operational Competitiveness Program – COMPETE, Partnership Agreement PT2020 (Research Unit 4293) and by National Funds through FCT under the project PEst-C/SAU/LA0002/2011. The research leading to these results has also received funding from the European Community's Seventh Framework Programme under grant agreement No. 602773 (Project KINDReD; Kinetoplastid Drug Development: strengthening the preclinical pipeline; HEALTH-F3-2013-602773). The COST Actions CM0801 “New drugs for neglected diseases” and CM1307 “Targeted chemotherapy towards diseases caused by endoparasites” have also contributed for this work.





## Author's declaration

Under the terms of the “Decreto-lei nº 216/92, de 13 de Outubro”, is hereby declared that the author afforded a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published articles included in this dissertation.

Under the terms of the “Decreto-lei nº 216/92, de 13 de Outubro”, is hereby declared that the following original articles/communications were prepared in the scope of this dissertation.

## SCIENTIFIC PUBLICATIONS

### Articles in international peer-reviewed journals

**Gaspar L**, Costa DM<sup>#</sup>, Pérez-Cabezas B<sup>#</sup>, Tavares J, Santarém N, Pruvost A, Ramos I, Pemberton IK, Thoo-Lin PK, Ronin C, Ciapetti P, Cordeiro-da-Silva A. Inhibitors of *Trypanosoma cruzi* Sir2 related protein 1 as potential drugs against Chagas disease (in preparation);

Campos JF, Cristo G, **Gaspar L**, Begouin A, Baptista CP, Queiroz MJRP, Cordeiro-da-Silva A. Synthesis of new 7-aryltioetherthieno[3,2-b]pyridines bearing arylamides or 1,2,3-triazoles 1,4-disubstituted and evaluation of their antiparasitic activity against *Leishmania infantum*, *Trypanosoma brucei* and *Trypanosoma cruzi* (in preparation);

Moraes CB<sup>#</sup>, **Gaspar L**<sup>#</sup>, Araujo-Junior AM<sup>#</sup>, Siqueira-Neto JL, Han D, Lee N, Cordeiro-da-Silva A, Schenkman S, Freitas-Junior L. High-Content Screening Assays for Chagas Disease Drug Discovery (in preparation);

Graça NAG<sup>#</sup>, **Gaspar L**<sup>#</sup>, Costa DM<sup>#</sup>, Loureiro I, Thoo-Lin PK, Ramos I, Roura M, Pruvost A, Pemberton IK, Loukil H, MacDougall J, Tavares J\*, Cordeiro-da-Silva A\* (2016) Activity of Bisnaphthalimidopropyl Derivatives Against *Trypanosoma brucei*. Antimicrob Agents Chemother (in press). doi: 10.1128/AAC.02490-15;

Cristo G, **Gaspar L**, Noro J, Baptista C, Thoo-Lin PK, Alves MJ, Cordeiro-da-Silva A (2015) Exploiting the Antiparasitic Activity of Naphthalimides Derivatives. International Journal of Chemistry and Pharmaceutical Sciences (in press);

**Gaspar L**, Moraes CB, Freitas-Junior LH, Ferrari S, Costantino L, Costi MP, Coron RP, Smith TK, Siqueira-Neto JL, McKerrow JH, Cordeiro-da-Silva A (2015) Current and Future Chemotherapy for Chagas Disease. Curr Med Chem 22(37): 4293-4312. doi: 10.2174/0929867322666151015120804;

Moraes CB, White KL, Braillard S, Perez C, Goo J, **Gaspar L**, Shackleford DM, Cordeiro-da-Silva A, Thompson RC, Freitas-Junior L, Charman SA, Chatelain E (2015) Enantiomers of nifurtimox do not exhibit stereoselective anti-*Trypanosoma cruzi* activity, toxicity, or pharmacokinetic properties. Antimicrob Agents Chemother 59(6): 3645-3647. doi: 10.1128/AAC.05139-14;

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## COMMUNICATIONS

### Oral communications

“Drug Discovery Strategies for Chagas Disease” in PhD training seminars, 16 of September 2015, at IBMC, Porto, Portugal;

Santarém N; Pérez-Cabezas B; Tavares J; Moreira D; Cecílio P; Baptista CP; Graça N; Ribeiro H; Faria J; Loureiro I; **Gaspar L**; Lima C; Amorim CG; Maciel J; Cordeiro-da-Silva A. “Pitfalls associated to the cultivation of Leishmania: a practical exemple” presented in COST Action CM1307 1<sup>st</sup> Conference/CaPF Joint Meeting, 26-29 of October 2014 at Corsica, France;

“Drug Discovery for Intracellular Parasites by High-Content Screening” in High-Content Imaging Screening at IBMC Workshop, 13 of January 2014, at IBMC, Porto, Portugal;

Poster communications

Cristo G, **Gaspar L**, Noro J, Duarte D, Oliva ACD, Baptista CP, Thoo-Lin PK, Alves MJ, Cordeiro-da-Silva A. “Exploiting the Antiparasitic Activity of Naphthalimide Derivatives” presented in COST Action CM1307 2<sup>nd</sup> Conference, 26-28 of October 2015, at Belgrade, Serbia.

Loureiro I, Costa D, **Gaspar L**, Graça N, Baptista C, Ribeiro H, Faria J, Tavares J, Cordeiro-da-Silva A. “Evaluation of the *in vivo* efficacy of bisnaphthalimidopropyl-derivative compound against *Trypanosoma brucei*” presented in 5<sup>th</sup> Advanced Summer School: Interrogation at the Biointerface – the Nano/Medicine Interface, 29 of June – 3 of July 2015, Porto, Portugal.

Loureiro I, Costa D, **Gaspar L**, Graça N, Baptista C, Ribeiro H, Faria J, Tavares J, Cordeiro-da-Silva A. “Evaluation of the *in vivo* efficacy of bisnaphthalimidopropyl-derivative compound against *Trypanosoma brucei*” presented in Joint Meeting of the 16<sup>th</sup> Drug Design & Development Seminar 2015 of the German Society for Parasitology & Workgroup 4 of the COST Action CM1307 “Targeted chemotherapy towards diseases caused by endoparasites”, 16-17 of March 2015, Berlin, Germany.



## Acknowledgments

This endeavor would not have been possible to complete without the guidance, help, support and friendship of several people that I met during the past four years, and that I wish to thank and acknowledge.

In first place, to my supervisor, Professor Anabela Cordeiro da Silva that provided me the opportunity to undertake the PhD in her lab and allowed me to participate in many projects. Also, she promptly established collaborations with other labs that deeply enriched this work. Her supervision, guidance, and support were fundamental to the success of this thesis.

To my co-supervisor Lúcio Freitas-Junior that received me at Institut Pasteur in Korea and introduced me to new scientific and cultural worlds that were highly enriching.

A very special thanks to four scientists that in many different occasions were determinant to the success of this PhD, by providing scientific guidance, experimental help, and uncountable hours reading and discussing the work herein presented. They are Carolina Borsoi Morais and Jair Lage Siqueira Neto at Institut Pasteur Korea and Joana Tavares and Nuno Santarém at IBMC.

I would also like to acknowledge the past and present members of the Parasite Disease Group that shared these four years with me: Gabriela Cristo, Nuno Graça, Ana Luísa Valente, Carla Lima, Helena Ribeiro, Célia Amorim, Inês Loureiro, Begoña Perez, Diana Moreira, David Costa, Renata Costa, Catarina Baptista, Joana Faria, Pedro Cecílio, Ana Teixeira, Mónica Sá, Joana Maciel, Tânia Meireles, Ricardo Silvestre, Vasco Rodrigues, Daniela Barros, Patrícia Varela, Joana Cunha and Dona Rosa that always kept the lab running smoothly. A thanks also to the remaining lab members of IPK, with a special acknowledgement to Miriam Giardini, Felipe Hilário and Kathrin Franzoi for their support and lasting friendship.

To Paola Minoprio and Sophie Goyard at the Trypanosoma Infectious Processes Group at the Institut Pasteur in Paris, as well as other lab members, that kindly hosted me and taught me many things about *Trypanosoma cruzi*.

## *Acknowledgments*

To my friends that all too often I neglected during these four years but whose friendship was essential in the good and not-so-good moments of this journey. Thank you.

And lastly, the most important thanks, to my family that always had time for me, even when I would not, and gave me unconditional support and comprehension in all the moments.



## Abstract

Human trypanosomiasis is a group of neglected tropical diseases caused by protozoan parasites of the genus *Trypanosoma*. *Trypanosoma brucei* is responsible for sleeping sickness, also called African trypanosomiasis, while *Trypanosoma cruzi* causes Chagas disease, or American trypanosomiasis. Together, they are responsible for significant mortality, morbidity and lost productivity in the endemic regions. There are no vaccines for these diseases, and treatments rely on drugs with limited efficacy, high cost, serious side effects and long administration periods. Since these diseases affect mostly the poor, there is no economic interest in the development of new drugs by pharmaceutical companies, and hopes for new treatments rely on public initiatives, public-private partnerships or philanthropic programs.

The first steps in the discovery of new drugs involve the identification of active molecules as starting points for further development, by either employing whole cells or specific molecular targets screenings. This thesis sought to exploit both strategies in the search for new molecules for trypanosomiasis drug discovery, with a focus on Chagas disease, one of the most neglected tropical diseases in the world.

Employing a phenotypic-based high-content approach on *T. cruzi* parasites infecting mammalian cells, it was possible to identify active and selective compounds among a chemical library composed of 4000 kinase/phosphatase-like inhibitors. Besides, a phenotypic profiling assay allowed the identification of a set of molecules able to interfere with the intracellular development of *T. cruzi* parasites. Using this phenotypic approach, it was also evaluated whether the efficacy of the racemic nifurtimox mixture could be improved by the separation of individual R- and S-enantiomers with different potency, toxicity or pharmacokinetic properties in various lineages of *T. cruzi*. However, no differences were found between isomers.

Silent information regulator 2 (Sir2) proteins, also called sirtuins, are a class of NAD<sup>+</sup>-dependent deacetylases conserved through evolution and present in all domains of life, from archaea to mammals. They are involved in many important functions such as gene silencing, DNA repair, longevity, metabolism, and others. Two homologues have been described in *T. cruzi*: a cytosolic Sir2 related protein 1 (TcSir2rp1) and a mitochondrial Sir2 related protein 3 (TcSir2rp3). A target-based drug discovery strategy was employed to characterize and evaluate the potential of TcSir2rp1 inhibitors as novel drugs for Chagas disease. It was found that *T. cruzi* Sir2rp1 has NAD<sup>+</sup>-dependent deacetylase activity. This activity was inhibited by nicotinamide, a classic sirtuins inhibitor. In addition, some bisnaphthalimidopropyl (BNIP) derivatives previously shown to inhibit the *Leishmania*

*infantum* orthologue (LiSir2rp1) also inhibited TcSir2rp1. Besides, they were active against intracellular amastigotes. The strongest inhibitor and most selective compound, BNIPSpd, was tested in an *in vivo* mouse model for Chagas disease but was ineffective in controlling the infection.

The potential of BNIPs as active molecules against *T. brucei* was also evaluated. One of the derivatives, BNIPDabut, is a potent inhibitor of *in vitro* growth of *T. brucei* bloodstream forms, although this activity is unlikely to be mediated by *T. brucei* Sir2rp1 inhibition, as demonstrated by biochemical assays. BNIPDabut had a strong *in vivo* antiparasitic activity in a mouse model of African trypanosomiasis, but without achieving cure. Nevertheless, BNIPDabut should be considered a scaffold for further development.

Keywords: *Trypanosoma cruzi*, *Trypanosoma brucei*, drug discovery, Sir2, bisnaphthalimidopropyl derivatives.

## Resumo

A tripanossomíase Humana é um grupo de doenças tropicais negligenciadas causada por parasitas protozoários do género *Trypanosoma*. O *Trypanosoma brucei* é responsável pela doença do sono, também chamada tripanossomíase Africana, enquanto o *Trypanosoma cruzi* causa a doença de Chagas, ou tripanossomíase Americana. Conjuntamente, são responsáveis por uma mortalidade e morbilidade significativa e por uma perda de produtividade nas regiões endémicas. Não existem vacinas para estas doenças, e o seu tratamento depende de fármacos com eficácia limitada, custo elevado, graves efeitos secundários e longos períodos de administração. Uma vez que estas doenças afetam maioritariamente populações pobres, não existe interesse económico das companhias farmacêuticas em desenvolver novos fármacos, e a esperança de novos tratamentos recaem em iniciativas públicas, parcerias público-privadas ou programas filantrópicos.

As etapas iniciais na descoberta de novos fármacos envolvem a identificação de moléculas ativas como base de desenvolvimento adicional, seja pela seleção contra células totais, ou contra alvos moleculares específicos. Esta tese procurou explorar ambas as estratégias na procura de moléculas para a descoberta de fármacos para a tripanossomíase, com foco na doença de Chagas, uma das mais negligenciadas doenças tropicais.

A abordagem fenotípica baseada em células mamíferas infetadas pelo parasita *T. cruzi* levou à identificação de compostos ativos e seletivos originários de uma biblioteca de compostos químicos constituída por inibidores de enzimas cínase e fosfatase. Adicionalmente, um ensaio de caracterização fenotípica permitiu a identificação de um conjunto de moléculas capazes de interferir com o desenvolvimento intracelular do parasita *T. cruzi*. Usando esta abordagem fenotípica foi também avaliado se a eficácia da mistura racémica do nifurtimox poderia ser melhorada através da separação individual dos R- e S-enantiómeros possuindo diferente potência, toxicidade ou características farmacocinéticas em diferentes linhagens de *T. cruzi*. Contudo, não foram verificadas diferenças entre os isómeros.

As proteínas pertencentes à família “Silent information regulator 2” (Sir2), também chamadas sirtuínas, são uma classe de enzimas com atividade desacetilase dependente de NAD<sup>+</sup> conservadas ao longo da evolução e presentes em todos os domínios da vida, desde as archaea até aos mamíferos. Estão envolvidas em várias funções importantes como o silenciamento genético, a reparação de DNA, longevidade, metabolismo, entre outras. Duas proteínas homólogas foram descritas em *T. cruzi*: a Sir2 “related protein 1”

(TcSir2rp1) citosólica e a Sir2 “related protein 3” (TcSir2rp3) mitocondrial. Uma estratégia de descoberta de fármacos baseada em alvos moleculares foi aplicada para caracterizar e avaliar o potencial de inibidores da TcSir2rp1 como novos fármacos para a doença de Chagas. Foi demonstrado que a Sir2rp1 de *T. cruzi* possui atividade desacetilase dependente do NAD<sup>+</sup>. Esta atividade foi inibida pela nicotinamida, um inibidor clássico das sirtuínas. Adicionalmente, alguns derivados do bisnaftalimidopropilo (bisenaphthalimidopropyl, BNIP) anteriormente caracterizados como inibidores da proteína ortóloga de *Leishmania infantum* (LiSir2rp1), também inibiram a proteína TcSir2rp1. Para além disso, os derivados foram ativos contra a amastigotas intracelulares. O inibidor mais potente contra a enzima e seletivo contra amastigotas, o BNIPSpd, foi testado num modelo *in vivo* de doença de Chagas em ratinho, mas foi ineficiente no controlo da infeção.

O potencial dos BNIPs como moléculas ativas contra *T. brucei* também foi avaliado. Um dos derivados, o BNIPDabut, demonstrou ser um potente inibidor do crescimento *in vitro* das formas sanguíneas do *T. brucei* apesar de esta atividade não ser mediada pela inibição da atividade enzimática da Sir2rp1 do parasita. O BNIPDabut possuiu forte atividade antiparasitária *in vivo* num modelo animal de ratinho de tripanossomíase Africana, mas sem cura total. Contudo, o BNIPDabut deverá ser considerado uma estrutura química líder para desenvolvimento adicional.

Palavras-chave: *Trypanosoma cruzi*, *Trypanosoma brucei*, descoberta de fármacos, Sir2, derivados do bisnaftalimidopropilo.

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## Abbreviations List

ADP – Adenosine Diphosphate  
 BNIP – BisNaphthallimidoPropyl  
 BSA – Bovine Serum Albumin  
 cDNA – complementary DNA  
 CDS – Coding Sequence  
 DALY - Disability-Adjusted Life Year  
 DNA – DeoxyriboNucleic Acid  
 DNDi – Drugs for Neglected Diseases initiative  
 DTU – Discreet Typing Unit  
 ELISA - Enzyme-Linked ImmunoSorbent Assay  
 FDA – Food and Drug Administration (American)  
 GPI – GlycosylPhosphatidyInositol  
 HAT – Human African Trypanosomiasis  
 HDAC – Histone DeACetylase  
 HTS – High-Throughput Screening  
 HYG – HYGromycin phosphotransferase  
 IFA – ImmunoFluorescent Antibody assay  
 Ig – Immunoglobulin  
 IGF-1 – Insulin-like Growth Factor-1  
 KAT – Lysine AceTyltransferase  
 kb – kilobase  
 KDAC – Lysine DeACetylase  
 kDNA – kinetoplast DNA  
 LaSir2rp1 – *Leishmania amazonensis* Sir2 related protein 1  
 LiSir2rp1 – *Leishmania infantum* Sir2 related protein 1  
 LIT – Liver Infusion Tryptose  
 LmSir2rp1 – *Leishmania major* Sir2 related protein 1  
 MHC – Major Histocompatibility Complex  
 MMS – Methyl MethaneSulfonate  
 mRNA – messenger RNA  
 mTORC1 – mammalian Target Of Rapamycin Complex 1  
 NAD<sup>+</sup> - Nicotinamide Adenine Dinucleotide  
 NADP – Nicotinamide Adenine Dinucleotide Phosphate  
 NEO – Neomycin phosphotransferase

## Abbreviations List

NES – Nuclear Export Signals  
NK – Natural Killer  
NLS – Nuclear Localization Signals  
NO – Nitric Oxide  
NOAEL – No Observed Adverse Effect Level  
OAADPr – 2'-O-acetyl-ADP-ribose  
PAC – Puromycin N-ACetyltransferase  
PAHO – Pan American Health Organization  
PCR – Polymerase Chain Reaction  
PfEMP1 – *Plasmodium falciparum* Erythrocyte Membrane Protein 1  
PfSir2A – *Plasmodium falciparum* Sir2 A  
PfSir2B – *Plasmodium falciparum* Sir2 B  
PTM – Post-Translational Modification  
rDNA – ribosomal DNA  
RNA – RiboNucleic acid  
RNAi – RNA interference  
ROS – Reactive Oxygen Species  
rRNA – ribosomal RNA  
Sir2 – Silent information regulator 2  
SM – Selection Marker  
T(SH)<sub>2</sub> – dihydrotrypanothione  
TbSir2rp1 – *Trypanosoma brucei* Sir2 related protein 1  
TbSir2rp2 – *Trypanosoma brucei* Sir2 related protein 2  
TbSir2rp3 – *Trypanosoma brucei* Sir2 related protein 3  
TcSir2rp1 – *Trypanosoma cruzi* Sir2 related protein 1  
TcSir2rp3 – *Trypanosoma cruzi* Sir2 related protein 3  
TCT – Tissue Culture Trypomastigotes  
TS – Trans-Sialidase  
TS<sub>2</sub> – trypanothione disulfide  
TSA – TrichoStatin A  
UTR – UnTranslated Region  
WHO – World Health Organization  
wt – wild type

# **PART I**

*Trypanosoma cruzi* and Chagas disease



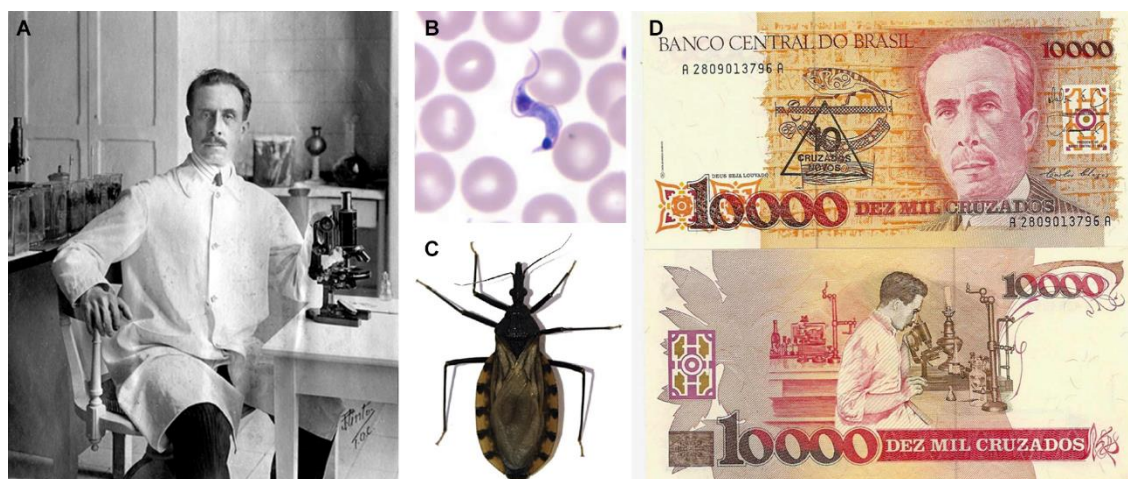
### 1.1. History and discovery

Paleoparasitological studies on mummies found in the north of Chile and southern Peru have proven that infection of humans by *Trypanosoma cruzi* goes back at least 9000 years [1]. The presence of the parasite itself largely pre-dates the arrival of humans to the American continent, some 15,000 years ago [2]. It is thought that the contact with humans first occurred when the early American nomadic people living a hunter-gathering lifestyle settled in valley and coastal regions of what is today the Atacama desert. The Chinchorros, as this people are known, probably perturbed the long established local sylvatic cycle of *T. cruzi* and thus were in the origin of human-vector transmission responsible for domestic cycle observed to our day. Deoxyribonucleic acid (DNA) analysis in more recent mummies and also in human remains from the Columbian era indicates that Chagas disease has been affecting human beings since then [1]. Probably the first clinical description of the disease was done by modern time physicians practicing in South America, like the Portuguese Luís Gomes Ferreira who described in 1735 the “bicho” disease characterized by an enlarged rectum, or the “mal de engasgo” (swallowing malady) in which patients had difficulties swallowing food [3]. These are probably accounts of the symptoms caused by the megavisceras characteristic of the chronic infection with *T. cruzi*. Charles Darwin, who has travelled to South America in 1835 describes a famous encounter with what was, at the time unknown for him, a vector species of the parasite. The journal entry for the episode reads: *“The night I experienced an attack (for it deserves no less a name) of the Benchuca (a species of Reduvius) the great black bug of the Pampas. It is most disgusting to feel soft wingless insects, about an inch long, crawling over one’s body. Before sucking, they are quite thin, but afterwards became round and bloated with blood, and in this state they are easily crushed. One which I caught at Iquique was very empty. When placed on a table, and though surrounded by people, if a finger was presented, the bold insect would immediately draw its sucker, make a charge, and if allowed, draw blood. No pain was caused by the wound. It was curious to watch its body during the act of sucking, as it changed in less than ten minutes, from being as flat as a wafer to a globular form”* [4].

It was not, however, until the pioneering work of Carlos Chagas (Figure 1 A) that the disease was fully characterized and understood [5]. The discovery happened in 1909 when Carlos Chagas, was sent on a campaign to provide aid as a malariologist to the workers constructing a new railroad in Lassance, Minas Gerais, Brazil. There one of the workers presented him with bugs that were biting them during the night, sucking blood, preferably in the area of the face. While examining one of these insects he detected trypanosome-like flagellated microorganisms, which he called *Trypanosoma cruzi* in honor of his mentor,

Oswaldo Cruz [5]. He then sent some of these insects to a research institute in Manguinhos, Rio de Janeiro, where they were allowed to bite monkeys. After about 30 days, the animals developed disease and had trypanosomes in their blood [5]. Carlos Chagas then returned to Lassance and started examining children with febrile symptoms. In one of them he found circulating trypanosomes with similar morphology to the ones in the blood of the monkeys, thus confirming the parasite was causing disease. Besides, he also describes finding circulating trypanosomes in a cat, suggesting they could be acting as domestic reservoirs [5].

Carlos Chagas was a brilliant physician and scientist. All by himself, he discovered the etiological agent (Figure 1 B), the transmitting vector (Figure 1 C), described the full life cycle and the main disease characteristics, as we know them today, in a period of just 5 months. In contrast, other parasitic diseases took years or decades to be completely elucidated, as well as more than one personality [6-8]. Although Carlos Chagas notable work was not awarded a Noble Prize (he was nominated two times) like some of his contemporary parasitologists, the Brazilians decided to honor him by printing bills with his image and his work, in one of those rare occasions where a biologist appears in a bank note (Fig. 1 D). Other names have been since then associated with the disease due to important discoveries, like Salvador Mazza that described the first case and the incidence of Chagas disease in Argentina [9], or his disciple Cecilio Romaña that gives the name to the characteristic Romaña sign but also many others that contributed to the present knowledge of *T. cruzi* and Chagas disease.



**Figure 1.** Historical features of Chagas disease. **A)** Dr. Carlos Chagas, the discoverer of the disease. **B)** Etiologic agent of Chagas disease, *Trypanosoma cruzi*. **C)** Natural transmission vector of the parasite, triatomines. **D)** Brazilian 10,000 cruzeiros bank note in honor of Carlos Chagas. [Adapted from [10-12]].

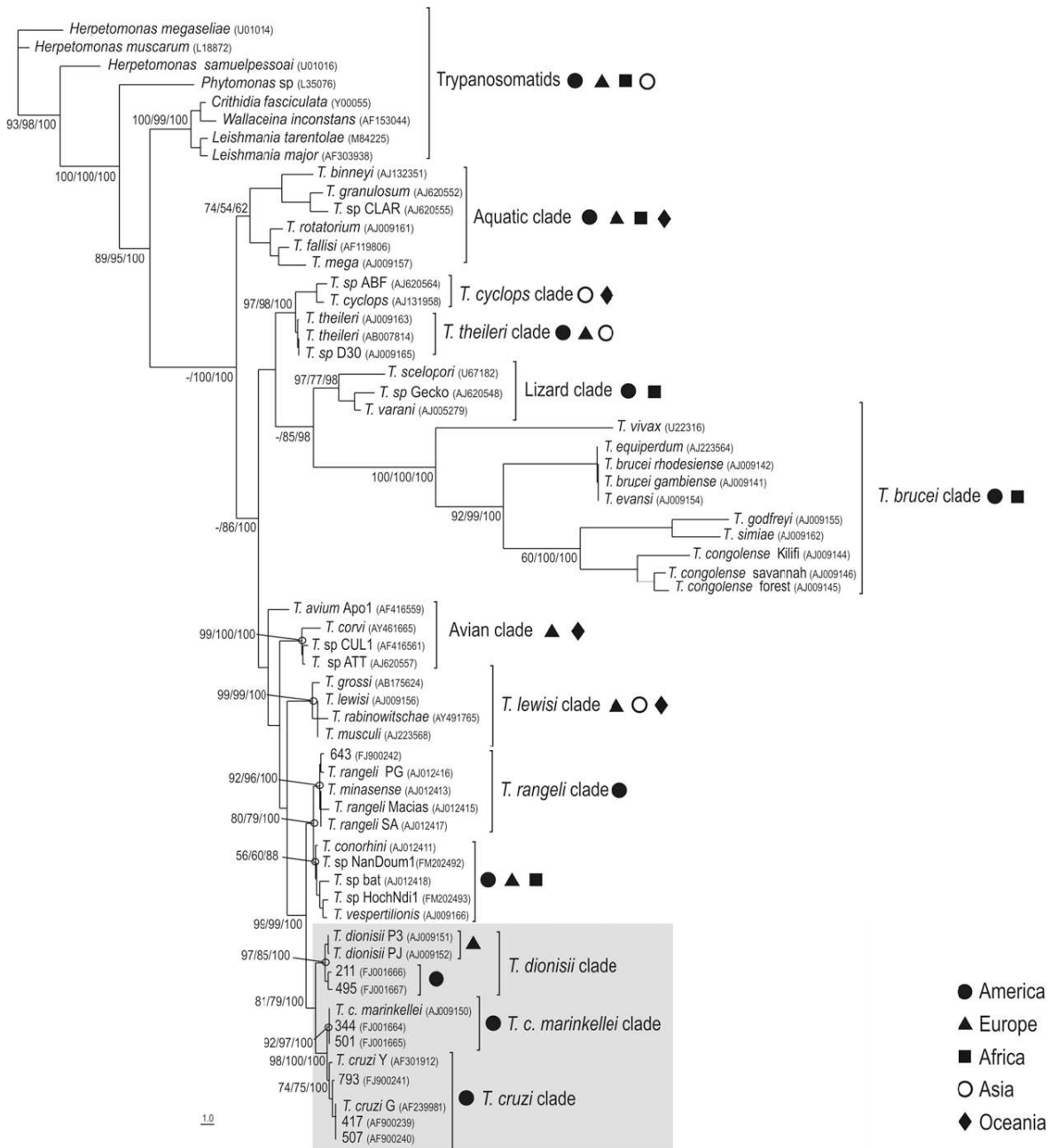


## 1.2. The species of *Trypanosoma cruzi*

Trypanosomes are early branching eukaryotes from the order Kinetoplastida, family Trypanosomatidae. They developed a very successful parasitism of all vertebrate animals, including fish, amphibians, birds, reptiles and mammals [13]. Moreover, they can be found in all the geographic areas of the globe [13] (Figure 2). All trypanosomes have a characteristic morphology in the vertebrate host bloodstream and their characteristic corkscrew-like motion was in the origin of their name, from the Greek *trypano* (drill, borer) and *soma* (body). The vast majority of trypanosomes are transmitted by arthropods, mostly insects [14].

The evolutionary origin of *T. cruzi* in particular, has been a topic of much debate. The first major theory, and the one widely accepted until recently, was the supercontinent theory. According to this hypothesis, the *T. cruzi* clade evolved separately from other African trypanosome (like the *T. brucei* clade) when the supercontinent formed by present day Antarctica, South America and Australia separated from Africa. Like many species in this continent, for example, primates, trypanosomes then began a distinct evolutionary process. This theory was supported by studies analyzing 18S ribosomal RNA (rRNA) suggesting that the clade to which *T. cruzi* belongs, the stercorarian trypanosomes (i.e. those transmitted by fecal contamination) has separated from salivarian Trypanosomes (i.e. those transmitted by bites, like the *T. brucei* clade) around 100 million years ago [15]. However, some discoveries have challenged this hypothesis. One of them is the apparent low diversity of species that does not seem to reflect at least 40 million years of independent co-evolution. In fact, no *bona fide* species of *T. cruzi* clade has been described to date. Another fact has been the detection of trypanosomes belonging to the clade of *T. cruzi* in the African continent [16]. Both pieces of evidence have questioned the validity of the continent isolation theory.

The hypothesis that is currently accepted is called the “bat seeding theory”. It considers that the common ancestor of the parasites belonging the *T. cruzi* clade was a bat trypanosome that diversified and spread geographically. Some of these trypanosomes subsequently switched from bats to terrestrial mammals, probably facilitated by an invertebrate vector that would feed on both hosts, and in one of these switches the *T. cruzi* species evolved. The theory is supported by the fact that the closest species of *T. cruzi* is *Trypanosoma marinkellei* found in South American bats [13] followed by *Trypanosoma erneyi* recently described in Mozambique, Africa [17] and by *Trypanosoma dionisii* found in bats both in the New and the Old World [18].



**Figure 2.** Phylogenetic tree of trypanosomes based on simple sequence repeats DNA showing the geographical distribution and variety of hosts trypanosomes parasitize. [Adapted from [13]].

### 1.3. *Trypanosoma cruzi* biology and unusual features

#### 1.3.1. Genome and gene expression

Although some characteristics had already been elucidated in the pre-genomic years, only after the completion of genomes sequencing of *T. cruzi*, *T. brucei* and *Leishmania major*, it was possible to characterize the full extent the particularities of the genomes of trypanosomatids [19-21]. The sequenced strain of *T. cruzi* (CL Brener), has a

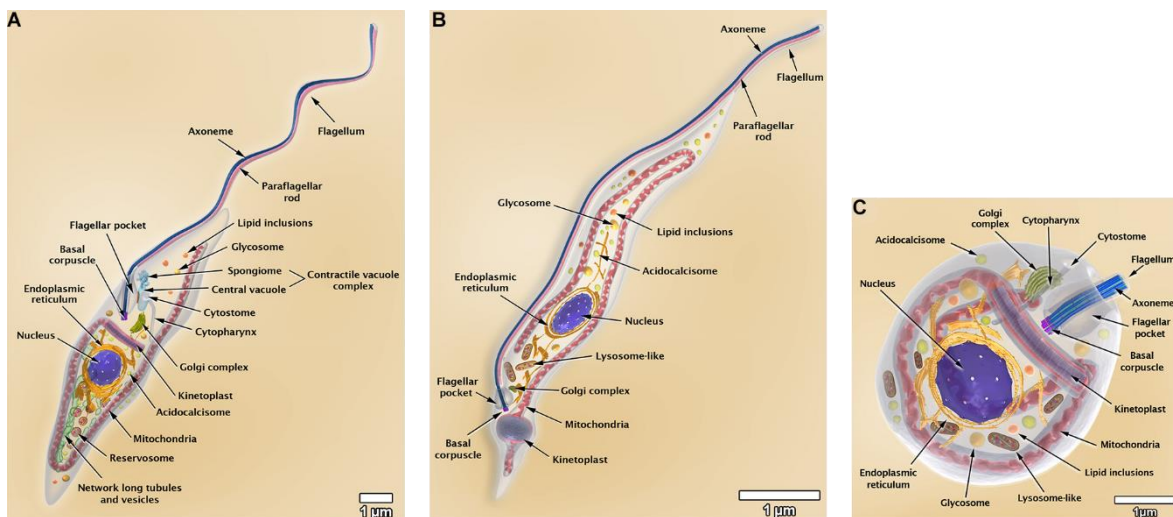
diploid genome size of about 110 Mb, with an estimated haploid gene number of 12,000 [19]. Because of the highly repetitive coding material of *T. cruzi*, accounting for about 50% of its genome, the chromosome assembly was not possible at the publication of the genome, and was later estimated to be 41 chromosomes [22].

Gene expression in kinetoplastids is polycistronic, a reminiscence of bacterial operons. Most protein-coding genes are organized in unidirectional, densely packed regions separated by strand switch regions. Transcription results in long strands of ribonucleic acids (RNA) containing 20 or more protein-coding genes that are not necessarily related. These molecules are afterwards subject to trans-splicing and polyadenylation, the former being a mechanism originally discovered in trypanosomes [23, 24]. This process of trans-splicing involves adding the same 39 nucleotide cap to the 5' end, called splice leader, to every mature messenger RNA (mRNA) in the cell. Unlike other eukaryotes, and because kinetoplastids do not possess introns, there is no cis-splicing, with the exception of four defined situations [21]. As a consequence of transcription's nature, the lack of evidence for differential regulation of RNA polymerase II and no identifiable RNA polymerase II promoter consensus sequence, gene expression regulation in trypanosomes is thought to be post-transcriptional [19]. The genes for most trypanosomal proteins are constitutively expressed in the different stages, nonetheless mRNA levels coming from the same polycistronic unit can vary significantly, suggesting an active control of mRNA half-life. Such a mechanism is essential to provide a fast adaptation, morphology modifications, surface composition and biochemical alterations necessary for the stage differentiations the parasite undergoes.

### 1.3.2. Kinetoplast

Kinetoplastidea have a single and highly ramified mitochondria containing a disk-like structure known as the kinetoplast. This structure is formed by a giant network of interlocked kinetoplast DNA (kDNA) rings resembling medieval chainmail [25]. These kDNA rings are called minicircles, of about 0.5 to 2.5 kilobases (kb) in size, and maxicircles, of about 20 to 40 kb, and exist in numbers of several thousands and a few dozen, respectively. Genes codified by the minicircles in kDNA are involved in a process firstly described in trypanosomes called RNA-editing. In this process, minicircles encode for guide RNAs that modify the products transcribed by maxicircles into functional mRNAs, in a process that inserts or deletes uridilate nucleotides [26]. RNA editing was later found to be also present in higher eukaryotes [27]. Maxicircles encode for transcripts similar in structure and function to those of mitochondrial DNA of higher eukaryotes, like rRNA and subunits of the

respiratory complexes [26]. Although kDNA seems to be essential for the completion of the digenetic life cycle of trypanosomes, it is noteworthy, however, that dyskinetoplastic trypanosomes can be found naturally, like a small portion of *Trypanosoma evansi* populations [28], or can be produced in the lab by chemical [29] or genetic manipulation [30, 31].



**Figure 3.** Schematic representation of *Trypanosoma cruzi* life cycle stages. **A)** Epimastigotes. **B)** Trypomastigote. **C)** Amastigote. [Adapted from [32]].

### 1.3.3. Glycosome

The glycosome is a characteristic organelle of trypanosome that seems to be a specialized form of peroxisomes, with similar biogenesis mediated by peroxins, of which there are more than 20 proteins [33]. The organelle contains enzymes of the glycolytic pathway that are usually found in the cytosol [34]. Thus, glycolysis in trypanosomes is bi-compartmentalized. The reason seems to be related with the fact that trypanosomes do not possess a mechanism of feedback inhibition by allosteric regulation like other eukaryotes, and must rely on the compartmentalization of relevant enzymes to prevent toxic accumulation of metabolic intermediates [35]. Additionally, it has been found that glycosomes possess many other enzymes required for other biosynthetic pathways, like peroxide metabolism, *de novo* pyrimidine biosynthesis and ergosterol biosynthesis [34]. Since the glycosome does not possess a genome, all the proteins it contains must be post-translationally imported from the cytosol [36, 37]. Proper biogenesis of glycosomes as well as the correct importing of glycolytic enzymes is essential to parasite survival [38].

#### 1.3.4. Trypanothione metabolism

In trypanosomes, like in any living organism, the homeostasis of redox equilibrium is affected by excessive reactive oxygen species (ROS) and nitrogen species coming from metabolism or the environment. The regulation of this intracellular redox state is mediated by thiol groups in proteins and low molecular mass compounds that act as redox buffers [39].

Whereas most organisms utilize glutathione and glutathione reductase as their major thiol-dependent mechanism, trypanosomes utilize a distinct system based on trypanothione and trypanothione reductase [40]. In *T. cruzi*, trypanothione is synthesized from one molecule of spermidine and two molecules of glutathione, in a reaction catalyzed by a single enzyme, trypanothione synthetase [41]. The active molecule dihydrotrypanothione  $T(SH)_2$  is a strong reducing agent, and upon reaction with oxidative species, is oxidized to trypanothione disulfide ( $TS_2$ ). Trypanothione reductase is responsible for reducing  $TS_2$  back to active  $T(SH)_2$  and thus maintaining an adequate pool of this antioxidant defense [42]. Although glutathione reductase and trypanothione reductase have a high protein homology, the latter is highly selective and is not able to use glutathione [43]. The intracellular role of trypanothione in trypanosomes is related with the delivery of electrons for the synthesis of DNA precursors in conjunction with the detoxification of hydroperoxides and is also important for other trypanothione dependent pathways [44]. Most of the enzymes in the trypanothione have been described as essential, and generated a high interest for the development of selective inhibitors as potential chemotherapeutics [45].

#### 1.3.5. Additional biologic features

Trypanosomes also possess acidocalcisomes, organelles that function as stores of calcium, magnesium, sodium, potassium, zinc, iron and also inorganic pyrophosphate and polyphosphate [46-48]. Additionally, they are also involved in pH homeostasis and osmoregulation of trypanosomes [48]. Originally discovered in trypanosomes, acidocalcisomes have also been described in other organisms and cells, including in the platelets [49-51].

The contractile vacuole is a structure that also plays a role in *T. cruzi* osmoregulation, by a mechanism of uptake or expelling of water in response to hypoosmotic and hyperosmotic stress, respectively [52].

The main component of the cytoskeleton of trypanosomes is a structure of microtubules that span the entirety of the body [53]. *T. cruzi* codifies for an actin protein, but no actin filaments have ever been seen in the parasite, and this is probably due to differences of homology in regions associated with oligomerization and interaction with actin binding proteins [20].

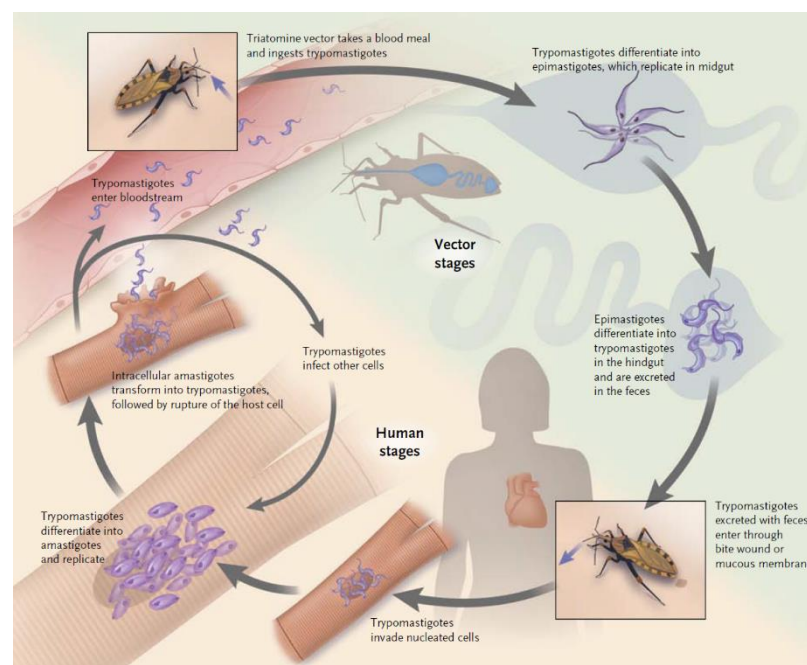
The single flagellum of trypanosomatids is present in all life cycle stages, even in amastigotes where it is shrunk to a residual structure. Like other flagella in other organisms, it is composed by a set of nine pairs of microtubules arranged around a central pair. It is a multifunction organelle responsible for cell propulsion, control of cell morphogenesis, with a role in directing cytokinesis and adhesion to host tissues [54-56]. The paraflagellar rod is a complex trilaminar structure linked to the axonemal microtubules of the flagellum and seems to have important functions for cell viability and function [57]. Recently, the disruption of two core paraflagellar proteins, PFR1 and PFR2, in the *T. cruzi* genome proved to be disruptive of the correct attachment of the flagellum to the cell body of epimastigotes [58].

The flagellum originates in a structure called the flagellar pocket. This is the place where exocytosis and endocytosis occur in trypanosomes, although it has been demonstrated that in *T. cruzi*, the cytostomes seem to be the main structure involved in the process [59]. It has important roles in cell polarity, division, trafficking and virulence [60].

#### 1.4. Life cycle

*T. cruzi* has a complex digenetic life cycle alternating between a mammalian host and a hematophagous insect vector. Remarkably, most of the features of the life cycle were discovered by Carlos Chagas in his seminal work, but new insights and refinements have been added in recent years [5] (summarized in Figure 4). Human infection begins when an infected vector takes a blood meal and subsequently defecates near the bite site. The feces of the insect are laden with infective metacyclic trypomastigotes (Figure 3 B) forms. Since the parasites are not able to penetrate intact skin, they must gain access to a lesion in the skin and the host usually does this when he scratches the bite. Alternatively, the parasite is able to directly invade mucosal tissues, like the oral or conjunctive mucosa. When the parasites penetrate the first external barrier, they infect local cells, usually resident macrophages or dendritic cells, being able to invade practically any nucleated cell [61]. Trypomastigotes enter the cell by either a mechanism of lysosome recruiting to the cell membrane, membrane expansion by actin-dependent mechanisms or by a plasma membrane invagination, all of which culminate in the formation of a parasitophorous vacuole

[62]. This vacuole eventually breaks up and the parasite is released to the host cell cytoplasm where it transforms into the amastigote - the replicative form in the mammalian host (Figure 3 C). After a few rounds of division, the amastigotes start to differentiate back to infective trypomastigotes. The trypomastigotes then rupture the cell and are released into the extracellular environment where they enter the blood or lymph circulatory system. These parasites are called blood trypomastigotes and are only found in the mammalian host. Although metacyclic and bloodstream trypomastigotes seem to have similar biological characteristics, like the invasion and the capacity to transform into amastigotes, they have some important differences in surface antigens and in overall gene expression profile [63, 64]. Through circulation, trypomastigotes gain access to other parts of the mammalian organism and re-infect a variety of cells. The parasite is transmitted to a vector by ingestion of blood with circulating blood trypomastigotes. In the midgut of the triatomines, the trypomastigotes differentiate into epimastigotes that are the non-infective and replicative form of the parasite in the vector (. After rounds of division, the epimastigotes migrate to the lower parts of the insect digestive track and in a new environment start differentiating to metacyclic trypomastigotes in a process called metacyclogenesis. These parasites are non-replicative and infective, being released to the exterior when the parasite defecates again after a blood meal, and thus closing the cycle.



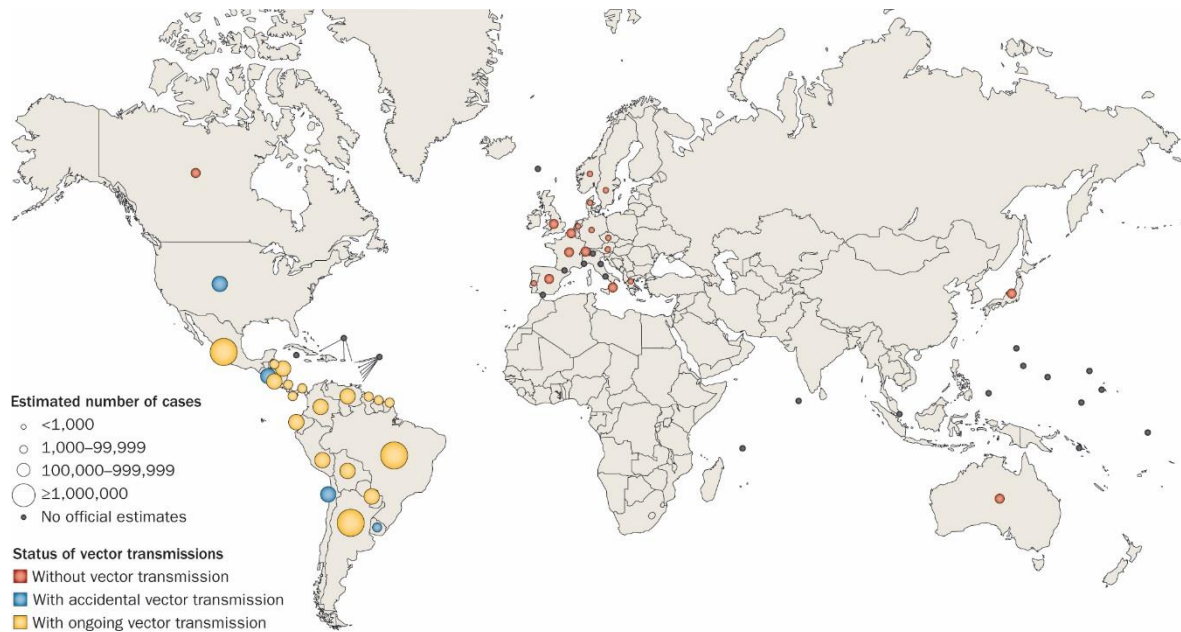
**Figure 4.** Representation of *Trypanosoma cruzi* digenetic life cycle between a triatomine and a human host. [Adapted from [65]].

## 1.5. Epidemiology

Chagas disease is the most important parasitic infirmity in the Americas, even considering that the region is also endemic for malaria and leishmaniasis. Recent estimates report the number of affected people to be around 6 million [66]. There are about 40,000 new cases every year, of which about one fourth are caused by congenital transmission, and the remaining by active vectorial transmission. Every year, Chagas disease claims more than 12,000 deaths [67, 68]. The population at risk is considered to be of 70 million people [66]. Disability-adjusted life years (DALYs) are an important measure of disease burden that expresses the number of years lost due to disability, poor health and early death. The number for DALYs for Chagas disease is a staggering 515 thousand years [69]. In fact, Chagas disease in the Americas is responsible for 8 to 10 times more DALYs than the aforementioned parasitosis malaria and leishmaniasis [69]. Considering that these people live already in underdeveloped regions, the affliction by the disease creates further hindrances to human development [70, 71]. The countries where the incidence is greater are Bolivia (6.1%) Argentina (3.6%), Paraguay (2.1%) and Ecuador (1.4) [66] (Figure 5). The reason for Bolivia to be the country most affected by Chagas disease is because there are more than a total of 8,000 new cases every year due to vectorial transmission alone. Mexico, despite having a lower rate of incidence of about 0.8%, still has more than 6000 of new cases every year. Brazil concentrates one of the largest numbers of infected individuals, of about 1.2 million people. This number has stabilized due to the interruption of the domestic vector transmission, and now the country only reports 46 new cases per year [66].

Although there are competent vectors and reservoir species of *T. cruzi* in the southernmost states of U.S.A., the incidence of the parasite in both vectors and reservoirs is very low. Coupled with high housing conditions in the country, vectorial transmission is almost inexistent. In fact, only 6 cases of vectorial transmission have ever been known to occur in USA [72]. Nonetheless, the country is home to about 300 thousand cases of Chagas disease due to the high number of people from endemic countries that has migrated and established there [73, 74]. Other non-endemic regions in the world also have imported cases of *T. cruzi*, most notably Canada, Europe, Japan and Australia (Figure 5). Spain, in particular, has a significant prevalence of *T. cruzi* because of the high number of Bolivian migrants living there [75].





**Figure 5.** Global geographic distribution of Chagas disease. [Adapted from [76]].

## 1.6. Transmission of Chagas disease

### 1.6.1. Routes of infection

The *T. cruzi* infection can be acquired by different routes. Historically, vectorial transmission has been the most important mechanism, from the ages of the first contact of humans with the parasite, until the present day [77]. This kind of transmission requires a particular ecological unit composed of domestic or sylvatic mammals and triatomine vector species susceptible to parasite colonization. Because no humans are required for the maintenance of the cycle that has evolved for millions of years, Chagas disease is considered to be a zoonosis where humans are accidental hosts.

The disease can also be transmitted from mother to son in a congenital manner. The incidence of congenital transmission is estimated to be around 1 to 10% of the newborn babies from mothers infected with Chagas disease [78, 79] (Barona-Vilar, 2012 #351, 80]. The transmission is transplacental, in a mechanism thought to first require the invasion of trophoblastic cells, followed by infection of adjacent tissues and finally the fetus [81]. Little is known about the factors that potentiate congenital transmission, except a correlation between levels of parasitaemia of the mother and the risk of infection to the fetus [82].

Chagas disease can also be acquired through the use of blood products and transplantation of organs. Concerning the blood products, platelets seem to be associated with a higher risk of infection, followed by blood [83]. This seems to be because platelets

end up being in the same centrifugation fraction of trypomastigotes. Because of the acquisition of many cases of Chagas due to this route, screening campaigns have been implemented, not only in endemic countries, but also worldwide due to the migrations from endemic countries that integrate local blood donation programs [73, 84, 85]. In the case of organs transplants, tissues of chagasic patients containing dormant or actively replicating amastigotes, once in a new host may establish a new infection [86]. The risks associated to transplants are many times increased because of the use of immunosuppressants (to control organ rejection) enabling unchecked *T. cruzi* replication due to the loss of immunological pressure [87].

Oral transmission is another source of Chagas disease, with 138 reported outbreaks responsible for the appearance of 776 new cases in the period of 2000 – 2010. Oral infection is usually acquired through ingestion of food or beverages such as juices, water or soup contaminated with *T. cruzi*-infected triatomines or their feces. It is thought that contamination takes place when the bugs that usually hide in the cavities of edible vegetation (e.g. sugar cane or açai berries) are accidentally poured during the preparation of these juices [88]. Some studies indicate that a very small inoculum is necessary to cause disease, because of the readiness with which laboratory animal become infected by this route [89]. Some reports have noted the increased aggressiveness of orally transmitted Chagas disease [90]. Early observations indicate that the mucin wall of particular strains of *T. cruzi* are physicochemically modified *in vivo* in way that the increases local gastric infectiveness [91].

### 1.6.2. Vectors

Natural transmission of *T. cruzi* is mediated by an estimated number 140 species of bugs belonging to the Triatominae sub-family of the Reduviidae family. They are grouped into five tribes: Triatomini, Rhodiini, Alberproseniini, Bolboderini and Carvernicolini, with the first two being the most important in terms of epidemiology [92]. Triatomines, commonly called kissing bugs because of their preference for biting in the face, are native to the American continent and have distinct species specific geographic distribution. They can be found in both South and Central America but also in the southern parts of North America, although vectorial transmission in the U.S.A. is extremely rare [93]. While some vector species are predominantly domestic and feed on humans, others are more associated with the sylvatic cycle, and only seasonally or opportunistically invade homes. The species with the most epidemiologic importance for human transmission are *Triatoma infestans*, followed

*Rhodnius prolixus* and *Triatoma dimidiata* [94]. While *Triatoma* is usually found in mud-walled houses or tiled roofs, *Rhodnius* is found in palm roofs. These distinct habitats seem to reflect the different evolution of these vectors and are also associated with the lineages of *T. cruzi* they predominantly are infected with: whereas *T. cruzi* lineage II has evolved with *T. infestans* in burrows and rocky grounds where edentates and ground dwelling marsupials co-habited, lineage I appears to have evolved with *R. prolixus* that inhabit palm trees where they feed on opossums [94].

Triatomines usually feed at night, when humans are physically inactive. They use a range of sensory systems to help them find their hosts, like CO<sub>2</sub> gradient, odors, moisture, heat and airflow, besides a sensitive infra-red detection [95]. The CO<sub>2</sub> of the breath is thought to be the reason why the bugs bite preferentially in the face. Sometime during or after the meal, they defecate the parasite laden fesses, in a time frame that varies from species to species, but is essential for effective transmission [96, 97]. Once they have taken the blood meal, they return to hide in the cavities and cervices during daytime, when hosts are physically active.

### 1.6.3. Reservoirs

Parasitic infection of the first mammalian hosts of *T. cruzi* are thought to have occurred about 80 million years ago [98]. The high diversity of hosts infected and the different environments where co-evolution occurred since then, allied with the many species of vectors aforementioned, are thought to be responsible for the heterogeneous nature of the different strains described to date.

The “reservoir” of *T. cruzi*, the non-human organisms that are infected with the parasite and actively transmit the parasite to the triatomine vector, is usually divided in sylvatic and domestic. In the sylvatic habitat, there is an estimated quantity of some 150 species and subspecies of animals that are infected with *T. cruzi* and can thus potentially act as reservoirs. Rodentia, Carnivora and Primates as well as orders of Marsupialia are among the many mammals described [99, 100]. The marsupial species and subspecies of *Didelphis* (common name opossums) whose ancestors are considered the first hosts of *T. cruzi* are also recognized as the most important reservoir because of its prolificacy, high adaptability and cohabitation with triatomines [101]. Interestingly, triatomines both feed on, and are part of the diet of opossums [100].

The domestic reservoir is formed by species that usually cohabit with human beings, either domesticated animals or undesired pests. Early reports have estimated the

percentage of *T. cruzi*-infected dogs and cats to be around 20 to 30% [102]. Recent studies have identified infected cats and dogs in all the Americas, even in the United States, and have estimated this prevalence to be 8-50%, much higher than the prevalence in the population [103, 104]. The actual contribution of infected cats and dogs in the dissemination of Chagas disease is however, still a topic of controversy. Another important peridomestic species is the common rat (*Rattus rattus*) with studies indicating presence of parasites in more than 30% of the animals evaluated [105]. Even if a distinction is made between sylvatic and domestic reservoir, it should be noted that both environments are dynamic and connected, since humans, infected animals and vectors can easily traverse them.

Because *T. cruzi* has such a large reservoir, most of which not quantified, total eradication of the disease is considered to be impossible.

### 1.7. Clinical manifestations

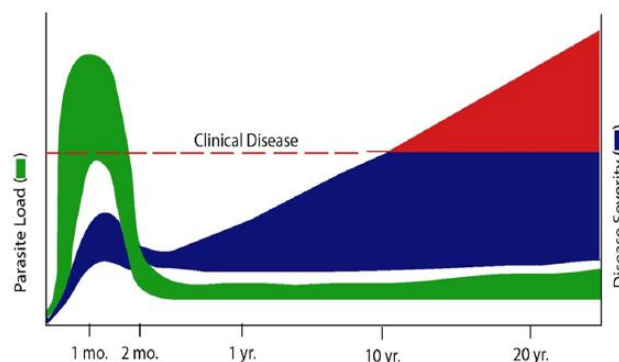
Chagas disease is generally divided in three major clinically distinct manifestations. The first phase of the disease is called the acute phase and is considered to occur 8 to 10 days after the infection by *T. cruzi*. It is accompanied by patent parasitaemia in blood and lasts for about 4 to 8 weeks. The big majority of the cases, more than 99%, are not diagnosed because of the absence of any symptoms, or the presence of mild, unspecific symptoms like fever or malaise (Figure 6). A rare sign of infection is the Romaña's eyelid signal or the chagoma that are swellings of the zone where inoculation took place, in the eye mucosa or other skin locations, respectively [106]. Both of them are frequently accompanied with an increase in volume of local lymph nodes. Meningoencephalitis and myocarditis are very rare manifestations that occur in less than 1% of the cases and are associated with a risk of death. In the vast majority of the acute phase cases all clinical manifestations disappear after approximately 60 days. Parasitaemias also are not detectable after this time, even if no targeted chemotherapy has been initiated, and affected individuals enter the indeterminate form of the disease. Spontaneous cure of the disease has been described, although it is considered to be rare [107].

Some differences are, however, verified when other transmission routes give origin to acute phase Chagas disease. Congenital acute phase babies commonly display, in addition, anemia and may also have a low birth weight or more serious consequences like abortion or neonatal death [77]. Oral acute phases and transplantation are other particular situations that have been described above. In the case of transplantation, because of the immunosuppressive therapies that are many times recommended with the transplant may

cause a particular aggressive progression of the disease with more serious consequences like acute myocarditis. The same is verified for patients co-infected with human immunodeficiency virus (HIV) [108].

The indeterminate form is defined by a positive diagnosis by serological or parasitological tests, associated with a completely asymptomatic profile. Only about 25 to 30% of these cases will eventually develop some of the chronic manifestations, while the remaining don't have any sign of the disease for the rest of their lives [106].

Apart from rare occasions where acute phase directly progresses to a chronic phase, the chronic manifestations of Chagas disease only appear years to decades later (Figure 6). In fact, many people only discover they are infected many years after the initial infection event. The chronic manifestations of Chagas disease are divided into cardiac, digestive or cardiodigestive forms, the latter being a mixture of the first two. The digestive forms affect 10 to 15% of the chronic patients with manifestation like megaesophagus and/or megavisceras. Such conditions are not usually life threatening and seem to be confined to countries like Argentina, Brazil, Chile and Bolivia, most likely as a consequence of a local infective strain [77]. Cardiac manifestations are the most serious consequences of Chagas disease present in 20 to 30% of the patients. The common clinical findings are arrhythmias and cardiac failure. Sudden death is the most common cause of mortality among chronic patients, being responsible for about two thirds of the deaths in this group [106].



**Figure 6.** Correlation between parasite load and disease severity over time in Chagas disease. [Adapted from [109]].

## 1.8. Host-parasite interactions

### 1.8.1. Innate and adaptive immunity in *T. cruzi* infection

One of the first lines of defense *T. cruzi* encounters upon penetrating the host organism is the complement system. Some antigens of *T. cruzi*, particularly trans-sialidases (TS), are recognized by complement elements, activating the complement cascade [110]. It has been demonstrated that trypomastigotes can activate the complement system by the lectin pathway and alternative pathway, whereas amastigotes are able to activate via non-classical pathway [111-113].

Some of the molecules of the parasite are also able to activate Toll-Like Receptors (TLRs), a family of membrane proteins mainly expressed in different immune cells that trigger innate immune responses against foreign pathogens [114]. Some examples are the glycosylphosphatidylinositol (GPI) anchors of mucins and Tc52 that activate TLR2 [115, 116], ceramide-containing GPI anchored molecules that activate TLR4 [117] and DNA from the parasite that activates TLR9 [118]. This last receptor has a central role in the control of acute infection, since mice knockout for Myd88 or TLR9 plus TLR2 fail to control the infection [119]. Moreover, the kinetics of TLR9 activation however, seem to be delayed [120], most likely because it takes time before DNA molecules originating from dead parasites are released. It is hypothesized that this lag period is enough for the parasite to establish a parasitaemia strong enough to avoid suppression by the immune system later on [120].

Neutrophils are the first cells to arrive to the parasite site of entry and uptake parasites that they kill by ROS and H<sub>2</sub>O<sub>2</sub> [121].

Macrophages arrive later, but have a central role in the immune response to the parasite. They act both as host cells to *T. cruzi*, but also as antigen presenting cells. Some of the antigens currently known to activate primed macrophages are tGPI, ssp4 and AgC10 [122-124]. The activation of macrophages also requires INF- $\gamma$ , the most important cytokine for *T. cruzi* infection control [125]. This activation enables the exertion of their antiparasitic activity by Nitric Oxide (NO) production intracellularly to kill amastigotes, but also possibly other mechanism to fight extracellular trypomastigotes [126-128].

Finally, DCs are at the interface of innate and acquired immune response and are some of the first cells to be readily infected by *T. cruzi*. They are initiators of adaptive immune responses and are more efficient activating naïve T cells [129].

Natural Killer (NK) cells are also present in the first line of defenses against the invading parasite. They are rapidly activated by cytokines produced by dendritic cells,

monocytes and macrophages that have contacted with the parasites becoming cytotoxic cells. They also help shaping the innate and acquired immune responses by interacting with T cells, macrophages/monocytes and also DCs. Experiments where NK cells were depleted from mice led to high parasitaemia and increased mortality [130]. In mice, NK cells are the major innate source of innate INF- $\gamma$  that macrophages recognize as a signal to produce NO and limit parasite replication during the acute phase [131].

T cell immunity also plays a role in the control of acute infection by both CD8<sup>+</sup> and CD4<sup>+</sup> mediated responses. In fact, knockouts for major histocompatibility complex (MHC) class II, CD8<sup>+</sup> or CD4<sup>+</sup> as well as anti CD8<sup>+</sup> and CD4<sup>+</sup> antibodies render mice highly susceptible to infection [132-134]. Whereas CD8<sup>+</sup> are more associated with cytotoxic activities, CD4<sup>+</sup> are responsible for INF- $\gamma$ -mediated activation of macrophages, induction of CD8<sup>+</sup> T cells, and stimulation of B cells to produce protective antibodies [135, 136].

As the disease progresses to a chronic phase, T cell immunity assumes the central role in keeping *T. cruzi* in check [137]. Some antigens of *T. cruzi* have already been characterized as being involved in acquired immune response stimulators, like the TS family [138], SA85-1.1 of TS [139], cruzipain [140] and KMP11 [141] through a MHCII mediated mechanism, and TSA1 [142], ASP 1 and 2 [143] and FL-160 [144] as MHCI-mediated.

B cells also have a role in acquired immunity. *T. cruzi* infection stimulates both a parasite specific antibody (mostly immunoglobulin (Ig) A and IgM) production in acute phase and a polyclonal B cell activation in the chronic phase (IgG that is maintained for life) [145]. TS, cruzipain and other antigens are thought to be the target of this type of immunity, particularly the SAPA repeat in TS molecules [146]. Polyclonal activation in the chronic phase is responsible by the production of large amounts of IgG that lack parasite specificity, and allow the parasite to survive by avoiding a specific response [147].

### 1.8.2. Evasion of the immune response

*T. cruzi* employs a range of mechanisms to evade the immune system and establish itself in the host organism as a silent pathogen. For this the parasites developed several strategies. One of them is the ability to escape the complement system. As described before, trypomastigote antigens are strong activators of the complement system, and thus the parasite needs to evade their deleterious effects. Some proteins have been described to be involved in the hindrance of complement activation by the alternative pathway, like gp160, gp58/68 and TDAF [148-150]. Also contributing to this inhibition is the presence of sialic acid on the parasite surface, harvested by the action of TS superfamily in host

molecules [151, 152]. *T. cruzi* is also known to shed C3b acceptors to the extracellular environment that also decrease complement activation at its surface. Both trypomastigotes and amastigotes have the capacity to resist complement lysis, however, epimastigotes do not [153].

The parasite is also able to escape from the action of antibodies. Cruzipain is a protease that is able to digest IgG molecules, both the Fab region in a lesser extent, and the Fc region in a major extent, thus disabling FcR binding [154].

When the parasites invade cells they must also protect from the intracellular aggressions of host cells. *T. cruzi* employs a range of antioxidant defenses like the trypanothione system, peroxidases and peroxiredoxins [155]. It has been demonstrated that stage differentiation from epimastigotes (insect stage) to metacyclic trypomastigotes (mammalian infective stage) is accompanied by an upregulation of antioxidant enzymes [156, 157]. Modulation of apoptotic responses has also been characterized in *T. cruzi* infected cells as a survival mechanism, namely the induction of early apoptosis by ceramide containing molecules of the parasite that assure premature and unharmed amastigote release [158]. In muscle cells, where *T. cruzi* is known to remain as a stealth parasite, it has been shown that there is less NO production and there are more polyamines that are thought to favor *T. cruzi* survival [159].

## 1.9. Diagnosis

The gold standard for Chagas disease diagnosis is the direct observation of parasites in blood smears [160]. However, because of the characteristic parasitaemia during disease progression, this method is mostly restricted to the acute phase, including congenital cases [161, 162]. Polymerase Chain Reaction (PCR) is also applicable to acute phase Chagas disease, however it is best suited for transplantation or accidental exposure situations [163].

Once the acute phase has run its course, the diagnostic tool that is most used is serology. Because parasites are, in most of the cases, not detected in the chronic phase, the most reliable diagnosis involves the detection of anti-*T. cruzi* antibodies, chiefly IgG. The serological tests available are Enzyme-Linked Immunosorbent Assay (ELISA), ImmunoFluorescent Antibody assay (IFA) and indirect haemagglutination [164, 165]. No single test is definitive, and confirmation should be obtained by two positive results, preferably with two different antigens (one from total parasite extracts and one recombinant antigen) [166-168]. Serology may be applied to diagnose children with acquired congenital



disease, but only after 9 months, time after which the mother antibodies disappear from the infant's body [169].

PCR diagnosis is not usually recommended for chronic disease detection because a negative PCR is not indicative of *T. cruzi* absence. It is not suited for routine diagnosis because of the need of specific labs, poor standardization of protocols and also variable results between labs [170, 171]. This technique may be, nevertheless, useful for situations of inconclusive serodiagnosis and as a readout of treatment progression. In fact, quantitative PCR has been elected the major assessment tool for treatment efficacy in a recent clinical trial for Chagas disease [172, 173].



# **PART II**

## Control of Chagas disease



## 2.1. Vectorial control

Vectorial control has played a very important role in the reduction of cases of Chagas disease. Multinational efforts like the ones led by World Health Organization (WHO) and Pan American Health Organization (PAHO) aimed at the elimination of the domestic vector populations are thought to be responsible for the decrease in cases from 24 million in the 1980s [174] to about 8 million cases in 2010 [66]. Today, countries like Brazil, Chile, Uruguay and Guatemala are considered to be free of the domestic vectorial transmission.

The control and eradication of domestic triatomine populations from many households and even entire regions has been achieved because of the use of effective chemical agents. There are many classes of insecticides, but the most used are pyrethroids like cypermethrin, cyfluthrin, deltamethrin and lambda-cyhalothrin. These neurotoxins alter the permeability of neurons to sodium and potassium ions, irreversibly depolarizing the membranes of the cell, and thus causing paralysis [175]. Resistance to pyrethroids has been found for *Triatoma infestans* [176] and it is thought that a suboptimal exposure to lethal pyrethroid concentrations selects eggs that are resistant to the insecticide and may display the resistance trait in the following developmental stages, including as adults [177]. A solution to this problem might be mixing or alternating the treatments with a different class insecticide. Other chemical-based strategies are the use of pyrethroid-impregnated curtains and bed-nets [178, 179].

Non chemical control methods can also be applied and are considered to be essential for the long-term elimination of vector infestation. Some of the most common measures are related to housing conditions improvements, like the substitution of palm thatched roofs by metal sheets and plastered walls instead of simple mud walls that can have cracks that harbor triatomines [180, 181]. Common in poor rural settings, these measures are a natural consequence of urbanization, where better construction and infrastructure planning are present. There have been reports, however, of cases where some triatomine species accompany the population and adapt to urban spaces, creating foci of Chagas disease transmission [182].

## 2.2. Therapies for Chagas disease

The contents of this section have been published in a review article:

**Gaspar L**, Moraes CB, Freitas-Junior L, Ferrari S, Costantino L, Costi MP, Coron RP, Smith TK, Siqueira-Neto J, McKerrow J, Cordeiro-da-Silva A. (2015) Current and Future Chemotherapy for Chagas Disease, *in Current Medicinal Chemistry*, 22(37): 4293-4312.

## Current and Future Chemotherapy for Chagas Disease

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**Abstract:** Human American trypanosomiasis, commonly called Chagas disease, is one of the most neglected illnesses in the world and remains one of the most prevalent chronic infectious diseases of Latin America with thousands of new cases every year. The only treatments available have been introduced five decades ago. They have serious, undesirable side effects and disputed benefits in the chronic stage of the disease – a characteristic and debilitating cardiomyopathy and/or megaviscerases. Several laboratories have therefore focused their efforts in finding better drugs. Although recent years have brought new clinical trials, these are few and lack diversity in terms of drug mechanism of action, thus resulting in a weak drug discovery pipeline. This fragility has been recently exposed by the failure of two candidates; posaconazole and E1224, to sterily cure patients in phase 2 clinical trials. Such setbacks highlight the need for continuous, novel and high quality drug discovery and development efforts to discover better and safer treatments.

In this article we will review past and current findings on drug discovery for *Trypanosoma cruzi* made by academic research groups, industry and other research organizations over the last half century. We also analyze the current research landscape that is now better placed than ever to deliver alternative treatments for Chagas disease in the near future.

**Keywords:** *Trypanosoma cruzi*, Chagas disease, benznidazole, nifurtimox, drug discovery, chemotherapy.

### 1. INTRODUCTION

Chagas disease is named after the Brazilian physician Carlos Chagas who first described the disease in 1909 [1]. Chagas disease is caused by the parasite *Trypanosoma cruzi* and is considered to be the parasitic infirmity with the largest social and economic burden in Latin America [2, 3]. There is an estimate of 7-8 million people currently infected with *T. cruzi*, while there are approximately 25 million people at risk of acquiring the disease [4]. Every year, Chagas disease

claims 10,000 deaths in endemic countries [5]. The parasite has a complex life cycle, alternating between the mammalian host and the hematophagous triatomine insect vector. The infection begins when the infected bug feeds on the host, which can be a wild or domestic mammal or a human. Infective metacyclic trypomastigote forms of *T. cruzi* are found in the feces of the bug which are released during the blood meal, and gain access through the lesion to infect dendritic cells [6]. Once inside a cell, the parasite breaks free of its entering organelle, the endosome/lysosome, and differentiates into a replicative amastigote form. The amastigote divides several times and matures into bloodstream trypomastigotes that rupture the host cell and are released into the bloodstream or lymph, free to infect a

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wide range of cells or be ingested by the transmitting vector, thus closing the cycle. In the triatomine gut, the parasite transforms once again into a replicative stage called the epimastigote and after clonal divisions it migrates to the final portion of the intestine and differentiates again into infectious metacyclic trypomastigote.

Although vectorial transmission has been greatly reduced due to vector control campaigns carried out by the World Health Organization (WHO), the Pan-American Health Organization (PAHO) and national health ministries of participating countries, there are still about 41,000 [5] new cases each year due to vectorial transmission. Many thousands of cases can be also attributed to secondary infection routes like transfusion of contaminated whole blood and derivate products, transplant of organs from chronically infected patients, congenital and oral transmissions. Oral transmission is a growing concern, with 138 outbreaks responsible for the appearance of 776 new cases in the period of 2000 – 2010. Oral infection is usually acquired through ingestion of food or beverages such as juices, water or soup contaminated with *T. cruzi*-infected triatomines or their feces [7]. Moreover, human migration in recent years has increased the incidence of new cases in non-endemic countries, making Chagas disease a public health issue in North America, Europe, Japan and Australia, requiring governments to implement screenings for blood and organs donations, as well as implement infrastructures to treat infected patients [8].

Due to the huge vertebrate reservoir and the variety of triatomine insects, the eradication of this zoonotic parasite is practically impossible [9].

The symptomatology of Chagas disease may vary according to the route of infection: while vectorial transmission is usually asymptomatic or presents non-specific symptoms, oral infection may increase the chance of acute cardiomyopathy because of the higher parasite loads associated. About 20 to 30% of acute cases develop serious chagasic cardiomyopathy with evolving symptoms and the risk of sudden death [10]. Fifteen to 20% develop digestive tract manifestations. The remaining infected individuals are considered to have the indeterminate form of the disease and the majority may not have symptoms or signs of the disease for all their life [10].

To treat new acute cases, indeterminate phase patients or reactivations from chronic patients, the only drugs available were introduced more than four decades ago, with no alternatives. Benznidazole and nifurtimox (Fig. 1A & B) are effective in treating acute infections, but efficacy is thought to decrease with the

disease progression, with little to no effect in the chronic phase. Additionally, they must be administered for long periods of time and display numerous side effects. Some of the most serious side effects require monitoring and ultimately, treatment interruption.

For the above reasons, new drugs to fight this disease are a dire need. New formulations of old drugs, old drugs with new applications as well as innovative drugs are feeding the pipeline for the treatment of Chagas disease [11].

In the following sections we will discuss the therapies available today and their limitations followed by the advances in the drug discovery and the candidates currently in preclinical and clinical studies to treat Chagas disease.

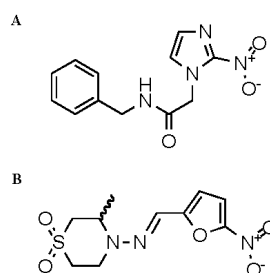


Fig. (1). Current drugs for Chagas disease: (A) benznidazole and (B) nifurtimox.

## 2. CURRENT THERAPIES

### 2.1. Benznidazole

Benznidazole (Fig. 1A) is a nitroimidazole (*N*-benzyl-2-(2-nitro-1*H*-imidazol-1-yl)acetamide) discovered in 1972 at Roche Laboratories, and originally marketed as Rochagan<sup>TM</sup> or Rodanil<sup>TM</sup>. Despite being introduced several decades ago, it is still the front-line treatment for the disease, although it is not approved by FDA [12]. Benznidazole is considered to be effective in reducing symptom severity and to shorten the clinical course and the duration of detectable parasitemia. Clinical cures are thought to be achieved in 60 to 85% of the acute cases and in more than 90% of congenitally infected infants, if treated in their first year of life [13]. Efficacy of benznidazole in chronic Chagas disease is still debatable, with reports varying from 15 - 35% of cure rates [14, 15]. The benefits of the drug in preventing cardiac and/or megacolon and megaesophagus manifestations were not clear for a long time [16]. To address this uncertainty, a large, multicenter, double-blind, randomized, placebocontrolled clinical trial



called BENEFIT (The Benznidazole Evaluation For Interrupting Trypanosomiasis, ClinicalTrials.gov, ID: NCT00123916) with 3,000 patients in several endemic countries has recently been concluded and demonstrated that a daily dose during 40 to 80 days of treatment failed in reducing mortality and morbidity in patients with chronic Chagas cardiomyopathy [17]. Problems with precise dosing in young children and the adverse effects observed has led to the development of a new pediatric formulation of benznidazole. This lower dose, easily dispersible tablet that should improve dosing accuracy, safety, and adherence to treatment is currently in clinical trials (Population Pharmacokinetics Study of Benznidazole in Children With Chagas' Disease - Pop PK Chagas, ClinicalTrials.gov, ID: NCT01549236). A previous clinical trial by the same team already validated the use of a lower dose benznidazole regimen for children aged 2 to 12 years old (Population Pharmacokinetics of Benznidazole in Children With Chagas Disease, ClinicalTrials.gov, ID: NCT00699387) [18]. Recently, a study on the pharmacokinetics of a new formulation of benznidazole (Abarax®, Elea Laboratories) in adults with Chagas disease also suggested that a dose reduction may be feasible [19].

The mechanism of action of benznidazole is thought to require the reduction of its nitro group by a trypanosomal NADH-dependent type I nitroreductase with generation of cytotoxic products that lead to thiol depletion and that can readily form adducts with many biological macromolecules like proteins, lipids and DNA that disrupt normal cell function and metabolism [20]. It is also thought that *T. cruzi* NADH-fumarate reductase inhibition, phagocytosis improvement and death by INF- $\gamma$  are additional mechanisms involved in parasite killing by benznidazole [21]. On the other hand, reduction by human liver NADPH, cytochrome P-450 reductase, P450, xanthine oxidase and aldehyde oxidase are thought to be responsible by the adverse side effects in patients [22].

While treatment with benznidazole is usually well tolerated by infants and children [23], adults frequently report adverse effects [24]. The most common side effect is dermatitis from hypersensitivity to the drug, appearing in up to 50% of the patients, usually after 10 days on the treatment, and for this reason, onwards weekly monitoring is recommended [25]. Digestive intolerance, peripheral neuropathy, depression of bone marrow, toxic hepatitis and lymphomas are other occurring side effects. Treatment interruption is most frequently due to dermatitis and digestive intolerance, al-

though studies reveal that low fat and hypoallergenic diet and daily dose administrations can reduce their incidence [25]. In addition, benznidazole should not be administered to pregnant women nor patients with severe renal or hepatic dysfunction, because of drug metabolism by these organs [26]. Exposure to the drug through breastfeeding is thought to be limited and unlikely to pose a risk [27].

Resistance to benznidazole has been reported under experimental conditions, however it is not yet clear how drug resistance correlates with benznidazole therapeutic failure in chagasic patients. An example is the Colombian strain, with benznidazole only being able to cure up to 16% of the mice infected with different clones [28]. *In vitro* results using real-time PCR suggest nitroreductases (NTRs) as the main mechanism of resistance *in vitro*, probably due to loss of a NTR gene copy [29]. A recent study warns of the relative ease in which benznidazole can develop resistance *in vitro* by a couple of different mechanisms such as chromosome loss and different point mutations in the NTR gene, all arising from a single population [30].

## 2.2. Nifurtimox

Nifurtimox ((R,S)-3-methyl-N-[(1E)-(5-nitro-2-furyl)methylene]thiomorpholin-4-amine-1,1-dioxide) a 5-nitrofuran derivative (Fig. 1B), constitutes the second and only alternative to benznidazole for the treatment of Chagas disease. Like benznidazole, the FDA has not yet approved chemotherapy of Chagas disease with nifurtimox. Also known as Bayer 2502, the drug, marketed as Lampit™, was originally discovered in that pharmaceutical company in 1965, exactly 50 years ago and provided, for the first time, a treatment for chagasic patients. It is also used in combination therapy with eflornithine to treat second stage Human African trypanosomiasis caused by the parasite *Trypanosoma brucei gambiense* [31, 32].

Nifurtimox efficacy is similar to benznidazole, but it has a much higher frequency of adverse effects [13, 26]. There is a frequency of adverse effects in 98% of patients, with only 56% of them completing the 60-day course treatment and 29% not tolerating it for more than 30 days. Digestive symptoms are predominant and neurological alterations the most persistent. An estimated 7% of patients had severe adverse effects like angioedema, myocarditis and grade-3 anaphylactic reactions [33].

Recently, a study highlighted the possible biochemical mechanisms that may be associated with

some of nifurtimox adverse side effects and as well as from other nitro aromatic derived drugs [34].

Nifurtimox acts through a mechanism of intracellular nitro reduction with the generation of the nitro radical, followed by redox cycling. In contrast, there is a greater role for oxygen reactive species, like superoxide ion and hydrogen peroxide which are toxic to *T. cruzi*. This parasite is sensitive to oxidative stress due to weak detoxification mechanisms due to the absence of catalase or peroxidase activity and reduced superoxide dismutase activity [21, 35]. RNA interference studies on *Trypanosoma brucei* show that besides NTR, other proteins linked to ubiquinone synthesis are also involved in nifurtimox mechanism of action in that species, and it is likely that the same mechanism is also present in *T. cruzi* [36].

Resistance to nifurtimox is readily obtainable *in vitro* and it seems parasite nitroreductases play a major role in its resistance, mounting up evidence that cross-resistance with benznidazole can occur as has been reported [37, 38], increasing the pressure to find alternative drugs to treat patients refractive to the only available therapies.

### 3. DRUG DISCOVERY AND DEVELOPMENT FOR CHAGAS DISEASE

#### 3.1. Drug Discovery Strategies

Various approaches can be adopted when considering the development of new drugs, and the field of neglected tropical diseases in particular has employed distinct strategies in the search [39]: (i) “*De novo* synthesis” is a classical way that focuses on the identification of new chemical entities through target discovery and compound screening. Although this is an important strategy to consider in the discovery of novel drugs for neglected diseases, it is a long-term approach. There are constraints like a high attrition rate of candidate compounds and the need for significant human and financial resources. Because of those issues and the perspectives of low market return and profits, the majority of companies do not make neglected tropical diseases a priority [40]. Populations affected by neglected diseases, and Chagas disease in particular, are economically poor and don't have the means to pay for expensive medication [41]. (ii) “Piggy-back” discovery is the name given to a process that takes advantage of prior research for an unrelated disease. It uses the data and chemical start points previously generated for a molecular target also present in the organism of interest. An example is the use of kinase inhibitor research data from cancer treatment to provide shortcuts for the de-

velopment of a kinase inhibitor against a parasitic target. (iii) Label extension or drug repurposing is the approach with the most immediate results, in that it uses already approved drugs for some pathologies, and repurposes them to be used in neglected diseases, saving considerable time and costs for approval processes after efficacy confirmation. Most of the toxicological data and often clinical tests are already available. An example of successful application of this strategy is the case of praziquantel for schistosomiasis and ivermectin for filariasis/onchocerciasis [39, 42]. More recently, auranofin, an approved drug for rheumatoid arthritis, has been identified as an amebicide [43] and a clinical trial is being launched in Bangladesh. So far, no drug has been repurposed for Chagas disease but some studies are devoted to find an extension for existing drugs employing both *in vitro* and *in silico* screening strategies [44, 45].

Independent of the strategy used, an essential feature of the drug discovery process that plays a guiding role is the target product profile (TPP). TPPs are a set of criteria to be followed through the development process and describe the needs and characteristic that the new candidate has to meet in order to constitute an improvement over the current available therapies. Drugs for Neglected Diseases initiative (DNDi), a non-profit drug research and development organization founded with the objective to develop therapies for neglected diseases, has recently updated a TPP for Chagas disease [46].

#### 3.2. Starting Points: Screening for Hits

Two different approaches can be taken to identify starting points for drug discovery: a molecular target or target-based approach, and a phenotypic approach, also known as untargeted drug discovery. Target based drug discovery relies on the previous discovery and characterization of a given molecular target and subsequent target validation by chemical or genetic means. Each has advantages and disadvantages [47]. Ideally, a target should be validated by more than one method. Chemical and biochemical validation is the proof that a molecular target, usually a protein, is able to be inhibited by a small molecule and that the use of such molecule in the parasite and/or *in vitro* and *in vivo* models of the disease leads to deficient parasite growth or ability to establish a normal infection. Genetic validation implies the reduction or elimination of the molecular target at the cellular level and the consequent observation of the interference in the parasite fitness/survival. The only reliable way to genetically validate a protein target in

*T. cruzi* is by gene knockout. Because of the low recombination potential of the parasite and slow growth kinetics, these transgenic techniques are extremely hard and time consuming to perform, with high failure rates and many weeks just to select stable transfected cells [48]. Unfortunately and unlike the related species *Trypanosoma brucei*, *T. cruzi* does not have functional RNAi machinery. Apparently some of its components have been lost or mutated during evolution [49, 50]. Most recently, CRISPR-Cas9 technology has been successfully applied to *T. cruzi* with major breakthroughs like reduction of gene expression of an enzyme gene family consisting of 65 members [51]. Chemical validation of a target requires a very specific and selective inhibitor or probe for a given target. Both genetic and chemical approaches therefore have limitations.

After drug target validation, biochemical assays can be set up to screen for inhibitors. These assays usually make use of proteins, frequently of recombinant origin. In the past, many of the labs working with *T. cruzi* have screened only a small number of compounds, from either synthetic or natural origin. The scenario changed dramatically with the development of high throughput technology based on assay miniaturization and automation of protocols, from procedure to analysis. This opened the door to large scale screening campaigns for targeting *T. cruzi*. Among the advantages brought by these innovations are the reduction of time to screen libraries, assay cost reduction and data reproducibility. As an example, one high throughput screen of 200,000 compounds against cruzipain, yielded 921 hit compounds that were subsequently screened by computational docking analysis to reveal 5 chemical scaffolds of common hits. These scaffolds are good starting points for further optimization and evidentiate the advantages of combining biological and bioinformatics analysis for prioritization of molecules after an high throughput screening campaign has been performed [52]. In another example of target based drug discovery, CYP51 from *Mycobacterium tuberculosis* was screened against a library of 20,000 organic compounds and resulted in two very active compounds [53], of which one (ChemDiv C155-0123) later showed selective inhibitory activity against the *T. cruzi* orthologous enzyme [54].

The limitations of molecular target approaches are the possibility of poor translation to a therapeutic effect, low druggability of the target, risk of off-target effects and the chance of redundant research by different groups since there are so few targets characterized [55].

To circumvent such limitations, phenotypic approaches have been developed. Instead of a single molecular target, whole cells are tested directly with the compounds and selection is made based on the observance of the required phenotype. This allows the selection of only those compounds that are active against the parasite, despite their mechanism of action. Also, it readily selects those compounds with the minimal pharmacodynamic and pharmacokinetic properties needed: proper intracellular distribution and accumulation, physiological binding and inhibition to target, etc., that are very difficult to predict with target based strategies. However, this method requires that the target must be elucidated in the discovery process, a task not always easy but achievable [56]. The most recent trend in whole cell assays has employed the use of high content screening analyzers – automated microscopes that can image many conditions (compounds) in clear bottom culture microplates. When the technology appeared one assay was developed that made use of mammalian cells expressing GFP and parasites stained with DAPI, and both manual and automated data analysis was performed [57]. Because of limitations of genetically modified parasites/host cells, another screening technology was developed that used unmodified cells and parasites. This assay was validated with a small library of FDA approved drugs [44]. The development of analysis software further automates the campaigns and allows the additional mining of important data, for example, the toxicity for host cells [58]. The first multi thousand screening campaign described in the literature has been recently published [59].

Balancing the benefits and disadvantages of both strategies in hit identification for parasitic diseases such as Chagas, phenotypic approaches emerged as the most promising methodologies [39]. Hits are selected for their ability to kill or not the parasite, coupled with cytotoxicity evaluation. When recent first-in-class new drugs, with innovative molecular mechanisms of action are tabulated, many of these were discovered by phenotypic screening (28 versus 17) [60].

### 3.3. From Hit to Lead and Beyond

Once single point hits have been obtained, the most promising are further validated in a dose response-curve to confirm activity. Most guidelines suggest an  $EC_{50}$  lower than 10  $\mu$ M as a good starting value, although recommendations can vary if other criteria are met, like a high selectivity index, for instance. The confirmed hits can also be subject to complementary activity assays. These can be of a different configuration,

employ a different readout, or even assess activity against other strains. A recent paper shows that a set of compounds in clinical trials have significantly different activity profiles depending on the strain they are tested on [61]. As has been discussed above, it is a requisite of the TPP for Chagas disease that a future drug is active against a large set of different DTUs. Another key unanswered question is whether a compound must clear the infection totally, as the reference compound, benznidazole, does. Does total clearance of infected cells, or parasites in animals or humans correlate with multistrain activity or, more importantly, with the clinical course of disease in the subsequent 20 years? Compounds that still meet agreed upon pharmacological and biological properties are called lead compounds. Leads are at the end of the screening campaign, but are the starting points of yet another phase in the drug discovery and development called lead optimization. In this phase, compounds enter a cycle of further testing, commonly with *in vivo* testing of activity, PK/PD, and toxicity. Leads are optimized by a medicinal chemist to try to improve potency, selectivity, reduce toxicity and enhance pharmacokinetic parameters [40]. The medicinal chemistry necessary for lead optimization is very costly and constitutes a bottleneck for many drug discovery efforts. Few in academia have the resources or access to the synthetic chemistry capacity necessary to produce the tens to hundreds of compounds usually required for lead optimization. Organizations such as DNDi and recently launched consortia based projects like the FP7 (Seventh Framework Program supported by the European Commission) KINDReD (Kinetoplastid Drug Development), NMTrypI (New Medicines for Trypanosomatidic Infections), PDE4NPD (Phosphodiesterase Inhibitors for Neglected Parasitic Disease) and A-PARADISE (Anti-Parasitic Drug Discovery in Epigenetics) have attempted to address this issue by coordination or outsourcing.

The optimized lead compound is one which can be called a pre-clinical candidate and enter the pre-clinical phase.

### 3.4. Animal Models

Animal models are used to extract the maximum possible information on drug efficacy and toxicity before testing the drug candidates in humans. Since the translation of data is of the utmost importance, several animal models have been studied to reproduce the physiopathology of Chagas disease. Models including mice, rats, rabbits, dogs and non-human primates have

been tried, but none of them completely mimics what happens in the human host [62]. The rat has been used in the past, but early observations concluded that it is somewhat resistant to *T. cruzi* infection, developing a mild and slow pathology [63]. Rabbits develop some of the chronic alterations such as focal myocarditis with a fibrous component, but do not show more severe forms of chronic myocarditis or the histological lesions in digestive track and skeletal muscles found in typical infections [64]. The Syrian hamster has also been proposed as an animal model for chronic Chagas cardiomyopathy but it was not able to reproduce all the characteristics findings of human cases [65]. Dogs, on the other hand, develop most of the clinical aspects of the disease found in humans. This includes modeling of the indeterminate stage of disease characterized by a latent infection without disease signs or symptoms and with normal electrocardiograms. Just a fraction of these animals develop chronic phase signs and symptoms [66, 67]. Monkeys are the closest phylogenetically related species to be used to study Chagas disease and results are more easily extrapolated to humans [67, 68].

Nevertheless, the mouse has remained the preferred animal model. Mice are easy to handle, house and are cheaper. Additionally, mouse models resemble many immunological, pathological and physiological aspects of human Chagas disease. One of the strains commonly used in experimental chemotherapy is Swiss mice, an outbred strain very sensitive to diverse *T. cruzi* genotypes [69]. Regarding inbred strains, Balb/C has also been extensively used and is considered one of the most susceptible to parasite infection in general [70]. C3H are a mildly resistant mouse strain commonly used to obtain chronic like infection in these animals [71]. C57BL/6 are considered to be among the most resistant strains, although susceptibility can vary widely depending on the strain of trypanosome used [72]. This genetic background is frequently used to obtain chronically infected mice in attempts to reproduce the pathophysiology of the chronic human disease.

Chronically infected mouse models develop an anti-inflammatory infiltrate and fibrosis in the heart, hallmarks of the disease in humans, but still lack extensive fibrosis, segmental myocardial abnormalities and macroscopic ventricle dilatation that closely resemble human chronic cases Chagas cardiomyopathy [65]. According to current protocols, four strategies have been employed to try to mimic chronic Chagas disease in mice: (a) a combination of susceptible mice strain, pathogenic *T. cruzi* DTU, age of animals and inoculation route that guarantees the survival of the animals to

the acute phase; (b) infection of mice with a lethal dose of *T. cruzi* followed by the treatment with a reference drug that assures animal survival, but not parasite clearance; (c) infection of resistant strains of mice with sub lethal inoculum of low pathogenic DTU; (d) infection of animals immunized by attenuated strains with a pathogenic DTU [73].

There are also dozens of different *T. cruzi* strains that have been used in animal models of the disease. Each research group works with a limited set of biological specimens that may reflect the history of the lab. An illustrative example of this variability is the case of A/J and C3H/HePAS mice infected with the same clone of Sylvio X10/4. Distinct histopathological findings are reported, suggesting a host genetic role in the manifestations and progress of the disease [74]. This variability hinders the extrapolation of results to other animal models and, ultimately, to humans.

Recent guidelines for *in vivo* testing of compounds in Chagas disease drug discovery have been elaborated. One protocol suggests three independent and consecutive *in vivo* evaluations of drug candidates: (1) testing for the effect of the compound on parasitemia reduction using Swiss female mice infected with Y strain, three doses of compound with the highest one set at the maximum tolerated dose, orally or intraperitoneally, and after five days of infection for a duration of five consecutive days; (2) analysis of parasitological cure during the acute phase using Swiss female mice infected with Y strain, with the dose established in the previous stage; (3) cure the acute phase of parasitemia caused by Colombian strain, which is benznidazole resistant [75]. Parasitemia is analyzed at 5, 8 and 10 days post infection (dpi) for (1) and (2) and at 20, 25 and 30 dpi for (3). Mortality is evaluated for all the three phases at 30 days and PCR, after immunosuppression with cyclophosphamide, to detect “latent” parasites. This technique was employed because it was proved to be more sensitive and time efficient than haemoculture. All the tests are done against a positive control of 100 mg benznidazole per kilogram of weight per day [75].

The effectiveness in the chronic and indeterminate stage takes place later in the development process, and the lack of it does not invalidate the prospective drug since, if the TPP is followed, it should be already an advance over existing therapies.

### 3.5. Biomarkers

Another obstacle in the drug discovery for Chagas disease has been the lack of reliable biomarkers of

cure. Traditionally, the definitive test of cure relies on conventional serology methods that have the limitation that it can take many years for the seroconversion to take place. Also, the majority of currently used methods employ crude antigen preparations from parasite life cycle stages not present in the mammalian host. Polymerase chain reaction is the standard method of cure in the current clinical trials and although useful, there is no proof of efficacy and it is only an indication of sterile cure for a given therapy [76]. Newer tests using recombinant proteins or peptides may be an improvement, but results are often inconsistent [77]. A recent and promising discovery in the field has been the identification of unusual fragments of human apolipoprotein A1 (APOA1) that are specifically present in chagasic patients and seem to disappear after treatment with nifurtimox [78, 79]. In mouse models, different methodologies to access parasitological cures were used after treatment with benznidazole and found out that even mice considered cured by hematological criteria still showed positive PCR tissues, either indicating a residual infection or presence of remaining parasite DNA [80].

## 4. TARGET CANDIDATES IN THE PIPELINE

### 4.1. Ergosterol Biosynthesis Inhibitors

Inhibitors of sterol 14  $\alpha$ -demethylase (CYP51) constitute a major fraction of all the drugs in the Chagas disease pipeline [11]. This enzyme is involved in the *de novo* synthesis of sterols in *T. cruzi*. While in mammals the major sterol is cholesterol, in plants, fungi and protozoa the major sterol present is ergosterol. The difference consists of a second double bond at the B ring and a fully saturated side chain with a methyl group at C24 in cholesterol [81]. CYP51 catalyzes a critical step of this biosynthetic pathway, removing the C14 methyl group from the sterol intermediate eburicol and originating 14 $\alpha$ -demethyl-14dihydroeburicol [82].

Ergosterol biosynthesis inhibitors are among the most common drugs used to treat fungal infections, and after the validation of this pathway in *T. cruzi*, compounds that were originally developed as antifungals were tested against the parasite. While some of the early generation imidazoles (*e.g.* miconazole, ketoconazole) and triazole (*e.g.* itraconazole, fluconazole) sterol biosynthesis inhibitors have some attenuating effect on the infection, they failed in achieving parasitological cures [83]. However, as newer azoles to treat fungi infections are still an ongoing interest for pharmaceutical companies, later generation drugs have also been tested for anti-*T. cruzi* activity.

One of the most promising molecules of the past decade was posaconazole (Fig. 2A). This triazole originally marketed as Noxafil by Schering-Plough pharmaceutical and active against *Candida spp.* and *Aspergillus spp.* is one example of the previously described drug repurposing strategy. Early assays demonstrated its potent and specific *in vitro* activity against *T. cruzi*, especially against the amastigote stage. Moreover, the effect on murine acute and chronic models was curative, rather than suppressive, as some earlier tested antifungal compounds demonstrated [84]. Later, posaconazole also proved to be an efficient trypanocidal against benznidazole and nifurtimox resistant strains, even in immunosuppressed mouse models, where the parasite would have a favorable environment to multiply [85]. A comparative study between posaconazole and benznidazole in a mouse model of Chagas disease showed both drugs led to 100% survival rates, suppression of parasitemia and negative *T. cruzi* antibodies. Only posaconazole-treated mice had completely negative haemocultures 54 dpi, whilst 50% of the benznidazole-treated had positive results. Also, plasma enzymatic assessment of cardiac lesion was indistinguishable from uninfected control for posaconazole, but significantly higher for benznidazole [86]. These promising results led to two clinical trials: one phase 2 trial sponsored by Hospital Universitari Vall d'Hebron Research Institute (ClinicalTrials.gov, ID: NCT00349271, CHAGASOL) that evaluated posaconazole and benznidazole for the treatment of Chagas disease chronic infection [87] and a phase 2 trial by Schering-Plough (now merged with Merck & Co.) for the treatment of asymptomatic Chagas disease, comparing a posaconazole with a placebo regimen and a combination of posaconazole with benznidazole (ClinicalTrials.gov, ID: NCT01377480, STOP CHAGAS) [88]. When evaluating posaconazole in patients, it is noteworthy that a woman with chronic Chagas disease and systemic lupus erythematosus requiring immunosuppression, was treated with posaconazole eliminating *T. cruzi* completely, whereas benznidazole treatment failed in this patient [89].

Unfortunately, the first clinical trial of posaconazole in humans did not replicate the results reported for the first patient. The treatment, consisting of two doses delivered orally for 60 days, had initial marked antitrypanosomal activity in chronic Chagas disease affected patients, but follow-up at the end of treatment suggested reactivation of infection, as documented by PCR. All but one patient treated with benznidazole showed negative PCR. The second clinical trial has finished in January 2015 and the final results should be

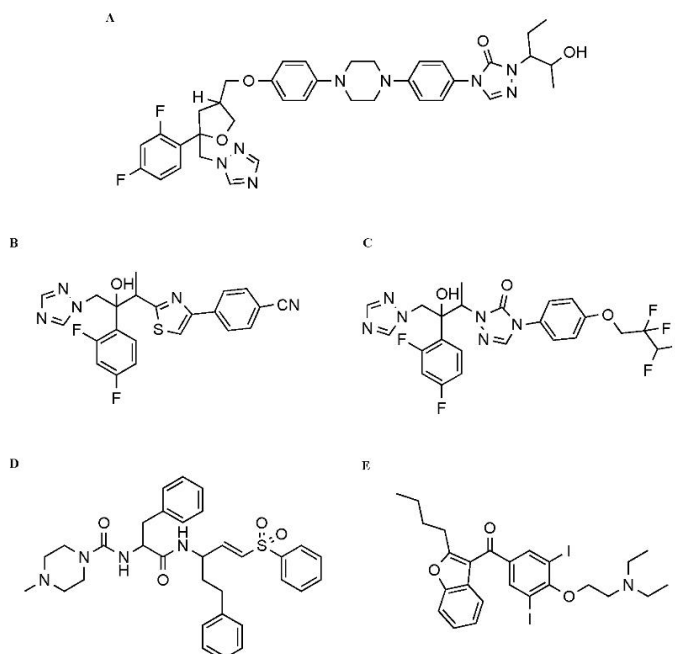
published soon thereafter. Moreover, two recent studies have demonstrated that benznidazole has a curative activity superior to that of posaconazole in newly developed mice models, suggesting that activity observed for a drug is highly dependent on the model used [90, 91].

Another ergosterol biosynthesis inhibitor in the recent pipeline was E1224 (Fig. 2B) from Eisai Co. E1224 is the monolysine salt of ravuconazole, thus a pro drug of an antifungal with a short half life. In this trial, which began in Bolivia in July 2011 as a partnership of DNDi and Eisai, adults with chronic “intermediate” Chagas disease were given placebo, E1124 or benznidazole (ClinicalTrials.gov, ID: NCT01489228). A series of examinations were then carried out in the following months in order to evaluate parasitological cures [92]. According to DNDi, the drug failed to maintain sustained efficacy 1 year after the end of treatment. The advantage of E1224, was that the structure of this compound was simpler and thus synthesis should be less expensive.

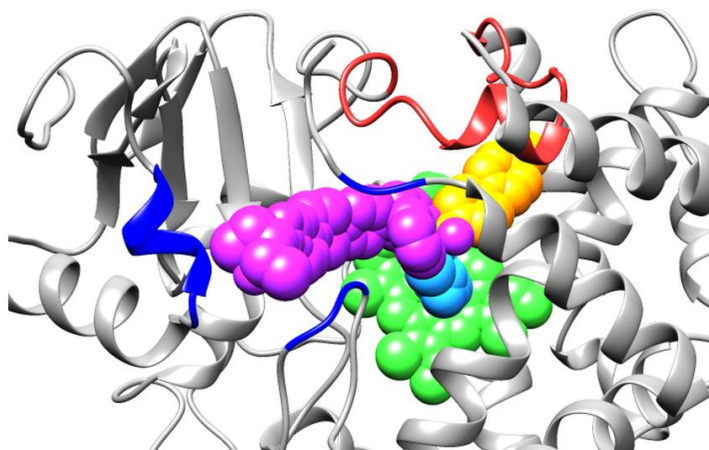
A third ergosterol biosynthesis inhibitor in clinical trials is Tak187 (Fig. 2C), a triazole synthesized in the 1990's and the property of Japanese Takeda Chemical Industries. There was 100% survival in the acute model of Chagas disease in mice treated with Tak-187 as well as a high parasitological cure (80%). In the chronic model, not all mice survived, but in those that did there was 100% parasitological cure even with benznidazole and nifurtimox resistant *T. cruzi* strains [93]. Subsequent studies confirmed that the drug could produce reductions of parasitemia similar to benznidazole, but at 10 times less dosage. Furthermore, it was superior to benznidazole in reducing inflammatory infiltrates and tissue damage in the heart and skeletal muscle of infected mice. The superior efficacy was attributable to higher intrinsic activity and long terminal half life [94]. Tak-187 has completed phase I trials [11].

The fungicide fenarimol, another inhibitor of CYP51, has been found to affect *T. cruzi* growth. After synthesis of analogues, the most promising compounds were tested *in vivo* in a Swiss mouse model with three dosing regimens. One analogue was effective in the 20 days regimen, reducing parasitemia to negligible levels that only reactivated after three cycles of immunosuppression [95].

While remaining controversial as to long term clinical effects, the failure of repurposed antifungals in Chagas disease clinical trials has focused attention to drug leads targeting the *T. cruzi* CYP51 (TcCYP51) itself [96, 97]. TcCYP51 is one of the most studied en-



**Fig. (2).** Drugs on the pipeline for Chagas disease: (A) posaconazole, (B) E1224, (C) Tak187, (D) K- 777, (E) amiodarone.



**Fig. (3).** TcCYP51 crystallographic structure (PDB ID: 3KSW). The protein is represented as a gray ribbon, the BC region that close the active site and includes residues important of the catalytic activity is colored in red, residues surrounding the entrance of the substrate channel are colored in blue. Inside the enzyme, the heme group is colored in green with atoms represented as sphere; the binding cavity is occupied by ligands (POZ (PDB ID: 3K1O) and VNF (PDB ID: 3KSW)), whose atoms are represented by sphere and colored as follow: purples the moieties that occupy the substrate channel; orange the moieties that occupy the deeper binding region; cyan the moieties involved in the interaction with the heme group.

zymes of *T. cruzi* as represented by crystallographic data for 18 structures of the protein with 16 different ligands in the protein databank (www.rcsb.org). Three structural features make this protein particularly interesting for a rational drug design approach: (i) high structure rigidity, particularly in its substrate binding cavity; (ii) a substrate access channel in both ligand free and bound structures that remains open and well defined; (iii) a substrate binding cavity that extends deeper inside the molecule than in other CYP structures [98]. Fig. (3) depicts the key structural regions of TcCYP51: the active site residues within the BC region (residues 100-120, PDB ID: 3KSW sequence numbering) that close the active site and isolate the substrate from solvent; the substrate tunnel through which substrate and ligands enter the active site; and the deeper substrate regions occupied by smaller ligands such as VNF and LFT (Fig. 4A & B) [97, 99, 100]. Some novel compounds designed to target TcCYP51 possess a nitrogen atom as a warhead, included in an azole or pyridine heterocycle that are able to form a coordination bond with the CYP51 catalytic heme iron and are represented in Fig. (4A-C). These compounds are simple and easy to synthesize and demonstrated strong inhibitory potential of intracellular amastigote growth of *T. cruzi* [97, 101-103]. VNI, in particular, was able to cure infected mice, has oral bioavailability and low toxicity, making it an excellent drug candidate [101].

Other enzymes of the ergosterol biosynthesis pathway may be targeted as potential drug targets for Chagas disease, including squalene synthase. This enzyme is responsible for the first step of ergosterol biosynthesis and was suggested as target in the parasites *Leishmania mexicana* and *T. cruzi* [104]. The effective and potent squalene synthase inhibitor 4-phenoxyphenoxyethyl thiocyanate effective against epimastigote proliferation producing an accumulation of mevalonate pathway intermediates is an example of compound targeting this enzyme [105, 106]. E5700, a drug from the Eisai Co. which is in development as human cholesterol lowering agent, is efficacious against *T. cruzi* [107]. Amiodarone (Fig. 2E) also inhibits ergosterol biosynthesis and is currently in clinical trials (as well as dronedarone) against the chronic phase of the disease [11]. This antiarrhythmic drug is used in the treatment of cardiac failure in chronic chagasic patients and has been found to act on a synergistic manner with azoles in disrupting *T. cruzi* biology. Amiodarone interferes with the calcium hemostasis but also inhibits ergosterol biosynthesis, while posaconazole or itraconazole also affects calcium hemostasis,

suggesting a viable and advantageous drug combination [108-110]. Allylamine terbinafine, a squalene epoxidase inhibitor, and mevinolin inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase, are antiproliferative against *T. cruzi* and both have been shown to be synergistic with ketoconazole against cultures of the parasite [111], suggesting they could be used in the treatment of human Chagas disease [112].

## 4.2. Cruzipain Inhibitors

K-777, a vinyl sulfone cysteine protease inhibitor was originally synthesized at Khepri Pharmaceuticals as an anti-inflammatory lead (Fig. 2D). It is an irreversible inhibitor of cruzipain, also known as cruzain or gp51/57. Cruzipain is a cathepsin L-like cysteine protease responsible for the majority of proteolytic activity in all the stages of *T. cruzi*. It may be essential for metabolism, metacyclogenesis, immune evasion, and invasion of host cells [113-116]. It has been suggested not only as a drug target but also as a vaccine target.

Early experiments with mouse models of Chagas disease showed that cysteine protease inhibitors were able to rescue mice from lethal infection, displaying repetitive negative haemocultures and so indicating parasitological cure [117]. K-777 was able to rescue mice from an acute and lethal *T. cruzi* infection even with a non functional immune system, as seen in immunocompromised patients (e.g. HIV/AIDS) or immunosuppressed individuals (e.g. transplantation patients) [118]. K-777 also abrogated myocardial damage in beagle dogs treated orally for seven days [119].

Several other classes of inhibitors of cruzipain have been reported as potential drug leads, including selenosemicarbazones [120], amidines bearing benzofuroxan or benzimidazole [121], and other scaffolds [122, 123]. Effective nitrile inhibitors of cruzipain have also been identified and serve to chemically validate this target [124].

A variety of approaches has been considered to rationally design inhibitors for cruzipain. Most of the compounds synthesized were originally designed to target the catalytic cysteine (Cys) of another member of the same enzyme family. The protein databank reports 24 crystallographic structures of cruzipain in complexes with inhibitors. They show that cruzipain is composed of one polypeptide chain folded into two domains: one mainly  $\alpha$ -helix and the other with an extended antiparallel  $\beta$ -sheet. The catalytic triad is Cysteine25, Histidine162 and Asparagine182. Together with the extended substrate binding site, they are found in the cleft between the two domains [125]. Within the



substrate binding site, different regions (S1', S1, S2 and S3), each devoted to the interaction with and binding of a residues of the peptidic substrate, have been recognized (Fig. 5) [126]. The S2 pocket, which has Glutamic acid 208 at the base is the key determinant for substrate specificity. This region adopts a substrate directed conformation if the S2 site is occupied by a basic or uncharged hydrogen bonding residues (such as Arginine and Tyrosine, respectively) whereas it assumes a solvent directed conformation when an hydrophobic residue (such as Phenylalanine) is bound [127]. Scaffolds designed to inhibit cruzipain include acylhydrazones, thiosemicarbazones and methoxyphenyl ketone derivatives. The discovery of acylhydrazone compounds as antiparasitic Cys protease inhibitors originated from an high throughput screening against brucipain, the related Cys protease of *T. brucei* [128]. Optimized scaffolds of this class of compounds have since been synthesized and also inhibited cruzipain (Fig. 4D) [129, 130]. Interestingly, acylhydrazones share some similarity with chalcones, in which the unsaturated arylketone subunit can act as a Michael acceptor (Fig. 6). Chalcones possess anti *T. cruzi* activity, but there was no direct evidence of cruzipain inhibition [131, 132].

A peptide vinyl sulfone (Michael acceptor) cruzipain inhibitor was able to cure parasitic infections in animal models, but with low oral bioavailability. An optimized compound derived from a methoxyphenyl ketone scaffold and with desirable physicochemical properties has been reported (Fig. 4E). Its mechanism of action, supported by the crystal structure of the complex, is depicted in (Fig. 7) [133].

Thiosemicarbazones (Fig. 4F) are another class of covalent inhibitors that originated from a screening of compounds able to inhibit cruzipain. Thiosemicarbazones inhibit Cys proteases through the formation of a reversible tetrahedral adduct of the Cys thiolate with the carbon of the thiocarbonyl group (Fig. 8) [134]. However, several members of this class of compounds were inactive versus the enzyme, yet were lethal to *T. cruzi* parasites [135-137]. This class of compounds was further modified according to the strategy represented in (Fig. 9) [138]. This led to compounds that targeted the parasite but were not cruzipain inhibitors. Compound G from (Fig. 4) inhibits cruzipain whereas its derivatives, as exemplified by compound in (Fig. 4H), did not. All exhibited strong antiparasitic activity. Because of the promiscuity of binding of thiosemicarbazones, this scaffold has been deprioritized because of concerns of off-target toxicity.

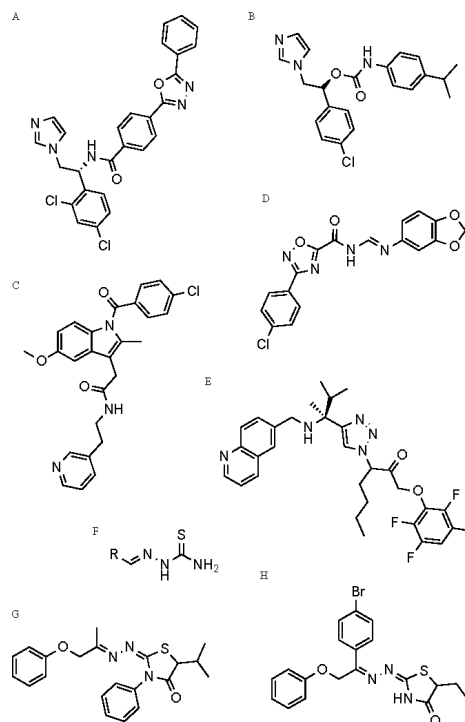
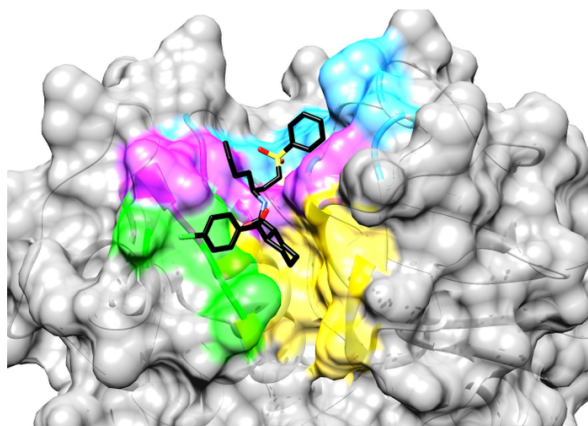


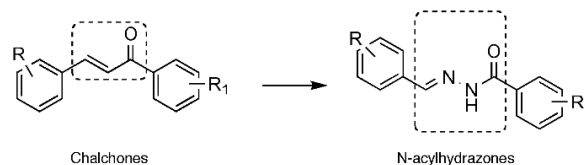
Fig. (4). Molecular structure of scaffolds currently being in study for Chagas Disease.

#### 4.3. Purine Salvage Inhibitors

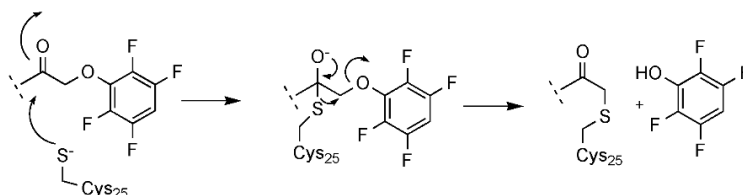
Allopurinol is an analogue of hypoxanthine that is used to treat gout, a condition characterized by deposits of uric acid in bone joints. The mechanism of action for this drug involves the inhibition of xanthine oxidase, an enzyme responsible for the consecutive conversion of hypoxanthine to xanthine and xanthine to uric acid. *T. cruzi* is not able to perform *de novo* synthesis of purines and needs to acquire them from the host. Since the microorganism does not possess xanthine oxidase, allopurinol is incorrectly sensed as a purine substrate and is directly incorporated in the parasite DNA by hypoxanthine-guanine phosphoribosyltransferase, disrupting DNA related processes. Previous assays showed the potential of the drug in arresting infection in cultured tissues [139], and a later study with other purine and pyrimidine analogues confirmed this activity [140]. In an animal model, allopurinol was able to reduce parasite blood levels, but with mild cardiac in-



**Fig. (5).** Crystallographic structure of cruzipain in complex with a non-peptidic vinylsulfone derivative (PDB-ID: 3HD3). The protein is represented as a surface colored in gray, specific sites are colored as follow: S1' in cyan, S1 in purple, S2 in yellow, S3 in green. The ligand is represented in stick, colored by atom as follow: C in black, O in red, N in blue, S in yellow.



**Fig. (6).** Similarity between chalcones and N-acylhydrazones.



**Fig. (7).** Mechanism of inactivation of cruzipain by a tetrafluorophenoxymethyl ketone derivative.

flammatory infiltrates at the heart. Altogether, the results demonstrated the drug modified the evolution of the infection and prevented the acute phase from evolving into chronic cardiac disease [141]. A comparative study between itraconazole and allopurinol in preventing chronic Chagas disease in Chile showed similar results in preventing cardiomyopathy, but itraconazole was preferred due to the fewer adverse effects [142]. A combination of allopurinol with clomipramine, a trypanothione reductase inhibitor, to treat Chagas disease in an acute mouse model was found to be no better than the use of clomipramine alone [143]. Although there has been an interest in the label extension of this drug,

early clinical evidence have discouraged the development of allopurinol as a drug to treat Chagas [144]. There is an interest in exploiting this pathway for Chagas chemotherapy, but to our knowledge no other compounds have been recently tested *in vivo* [145]. Several 4'-substituted and 3',4'-disubstituted 5-benzyl-2,4-diaminopyrimidines are selective inhibitors of *T. cruzi* dihydrofolate reductase and showed good *in vitro* activity against the parasite [146].

#### 4.4. Inhibitors of Pyrophosphate Metabolism

Another pathway that has gained attention is the one responsible for pyrophosphate metabolism. This proc-

ess does not take place in the cytosol but rather at acidocalcisomes, parasite specific organelles that are also involved in calcium hemostasis, response to cell stress, osmoregulation, and energy transduction [147]. It has been demonstrated that bisphosphonates, drugs currently used to treat osteoporosis, accumulate in the acidocalcisomes and can inhibit a key enzyme of pyrophosphate metabolism – farnesyl pyrophosphate synthase [148].

The first report of this activity in animal models demonstrated that risedronate could reduce parasitemia with reductions in mortality, but no complete parasitological cures were achieved [149]. In another study, a significant reduction in mortality was observed when CD-1 mice were treated with risedronate, but myocardial pathology and ventricular dilatation was unchanged in comparison with control. On the other hand, Tulahuen strain infected C57BL/6 mice had no improvement in mortality [150].

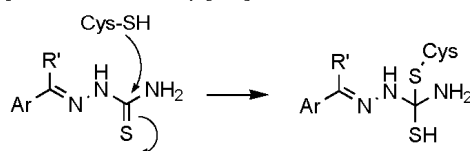


Fig. (8). Mechanism of inactivation of Cys proteases by thiosemicarbazones.

More recently, metal complexes of the bioactive, bisphosphonates alendronate [151] pamidronate [151] and risendronate [152] were synthesized and showed activity against amastigotes, with no toxicity for the mammalian host cells tested. These complexes are thought to protect phosphonate groups from ionization at physiological pH, increasing bioavailability to target the parasitic farnesyl pyrophosphate synthase [151]. Newly synthesized bisphosphonates also proved to be potent inhibitors of *T. cruzi* farnesyl pyrophosphate synthase [153].

#### 4.5. Trypanothione Biosynthesis Inhibitors

Instead of glutathione and glutathione reductase, trypanosomatids produce trypanothione and trypanothione reductase for thiol-dependent redox metabolism. This is essential in detoxification of free radicals and maintenance of the intracellular reducing environment. As this parasite specific system does not exist in the humans, it is considered a potential target [154]. Recently, a study validated the trypanothione pathway as drug target by a metabolic modeling approach. All of the constituent enzymes and transporters present are essential for proper pathway function, but not all of them have therapeutic potential [155]. Many reductase inhibitors have been identified, including polyamine derivatives, crystal violet, acridine based tricyclics, phenothiazine, benzoazepine, isoalloxazine and pyridoquinoline [156-159]. *In vivo* testing of a trypanothione reductase inhibitor, thioridazine, promoted heart protection but failed to completely eradicate the parasite [160].

#### 4.6. Lipid Biosynthesis Inhibitors

Alkyllysophospholipids and lysophospholipid analogues, such as miltefosine and edelfosine have been shown to be active against proliferation and differentiation of *T. cruzi*, *in vitro* and *in vivo* with good oral activity and low toxicity [161-166].

Surprisingly little is known about the role of glycosphingolipids in trypanosomatids. Various glycosphingolipids inhibitors have shown antiproliferative activity lysing 79 to 95.5% of parasite in an *in vitro* assay and cytostatic activity in mouse model [167].

Although *T. cruzi* glycosphosphatidylinositol GPI anchors share the same conserved core as other eukaryotes, many often contain a fourth mannose on which resides a galactofuranosyl (Gal/) linked to the O-3 and an obligatory 2-aminoethylphosphonate (2AEP), also known as ciliatine, linked to O-6 of the glucosamine of all *T. cruzi* GIPLs and GPI-anchored mucins

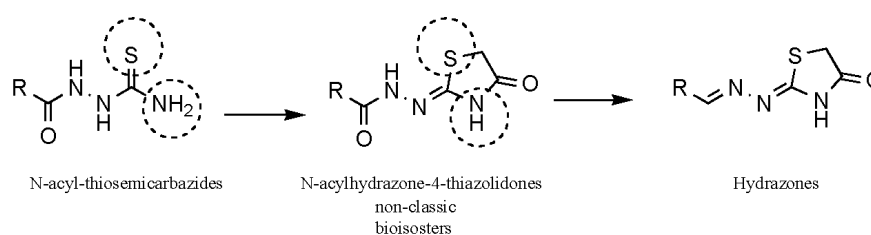


Fig. (9). Design of hydrazones as anti *T. cruzi* compounds.

[168-170].

Enzymes involved in the biosynthesis of the *T. cruzi* GPI anchors are susceptible to inhibitors in different organisms [171, 172]. A recent study identified the orthologous sequences of all genes involved in the biosynthesis of the *T. cruzi* GPIs. Three of these sequences complement yeast conditional mutants of these homologues. Unsuccessful attempts to generate *T. cruzi* knockouts for three of these genes further suggested that the GPI is an essential component of the organism [173].

Although the role of AEP in *T. cruzi* has not yet been identified, it is a virulence factor in pathogens like *Bacteroides fragilis* [174]. The absence of the AEP biosynthetic enzymes (phosphoenolpyruvate mutase, phosphoenolpyruvate decarboxylase & 2-aminoethyl-phosphonate transaminase) in humans make the pathway a potential candidate for drug targeting. Recent investigations by Coron and Smith (unpublished) have genetically validated the AEP biosynthetic pathway. They identified compounds with potent *in vitro* activity against the recombinant enzymes of the AEP pathway as well as against *T. cruzi* epimastigotes.

## CONCLUSION

Since the discovery of *Trypanosoma cruzi* as the etiological agent of Chagas disease more than 100 years ago, a cure has been pursued. It took 50 years to discover a specific drug, nifurtimox, to treat this neglected disease. However, apart from the discovery and development of benznidazole some years later, no improvements in chemotherapy have been made in safety or effectiveness, despite a half century of intense research. Nevertheless, there are reasons to be optimistic. The global nature of the disease and the information about its pathology, has brought new researchers to the field. Novel basic and applied research is constantly feeding our knowledge of the disease. New partnerships, including with pharmaceutical companies, are accelerating efforts traditionally made solely by academia. Clinical trials for some candidates have been recently completed, some are under way and a few more are planned to begin shortly. But we should not ignore the still long way and challenges to find a better treatment for Chagas: resources and funding are scarce, and there is a critical need to define beneficial intellectual property agreements and improve data sharing for all involved in Chagas disease drug discovery.

## CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

## ACKNOWLEDGEMENTS

Luis Gaspar is thankful to FCT for funding (scholarship reference: SFRH/BD/81604/2011). The research leading to these results has received funding from the European Community's Seventh Framework Programme under grant agreement No.602773 (Project KINDReD), No. 603240 (Project NMTrypI) and COST Action CM1307.

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Received: ?????????

Revised: ?????????

Accepted: ?????????

# **PART III**

**Sirtuins: novel targets  
for parasitic diseases?**



### 3.1. Histone deacetylases and the class of sirtuins

Post-Translational Modifications (PTMs) represent one of the major mechanisms in regulating protein function in all life forms. Through phosphorylation, acetylation, methylation, glycosylation and ubiquitylation, cells greatly extend the possibilities beyond the coding genome [183]. PTMs can change the enzymatic activity of a protein, change its subcellular localization, interfere with interaction partners, increase or decrease its stability and induce DNA interaction [183, 184].

Discovered half a century ago and largely ignored for the following years, lysine acetylation has re-emerged in the last two decades as a highly important PTM. It has been placed by some authors in the same level of biological relevance as phosphorylation [185, 186]. In fact, studies of the acetylome (the acetylated-proteome) of mammalian cells, revealed acetylation sites in 1,750 different proteins [187], a number close to the about 2,000 proteins found to be phosphorylated [188].

The “acetyl code” is maintained by three different protein types: the “writers”, lysine acetyltransferases (KATs) that add acetyl groups to proteins, the “erasers”, lysine deacetylase (KDACs) that remove acetyl groups, and “readers”, proteins that specifically recognize and bind acetyllysine groups [189].

KDACs in particular have been the focus of great interest by the scientific community due to their many roles in cell function and disease. KDACs are interchangeable called histone deacetylases (HDACs) because the first reactions catalyzed by these proteins to be discovered were the removal of acetyl groups in histone tails [185, 190].

HDACs are separated in four different classes based on sequence homology (Class I, II, III and IV) and two different families: the histone deacetylase family and the sirtuin family, the latter being all class III HDACs. While the first family has a limited set of molecular targets mainly composed of histones, sirtuins have a variety of substrates ranging from metabolic enzymes to structural proteins as well as histones [191-193]. The sirtuin family seems to be ubiquitous throughout all kingdoms of life. The number of genes coding for sirtuins within an organism ranges from as little as one in bacteria to seven in vertebrates [194]. The sirtuin family is further classified in 5 subclasses (I, II, III, IV and U) [195].

## 3.2. Enzymatic activity of sirtuins

### 3.2.1. NAD<sup>+</sup>-dependent deacetylase activity

The most common reaction catalyzed by sirtuins is deacetylation. This reaction is of utmost biological importance as there is a clear relation between the acetylation status of several proteins and their cellular functions [196-198]. The deacetylase reaction requires (nicotinamide adenine dinucleotide) NAD<sup>+</sup>, an acetylated lysine residue and produces deacetylated lysine, nicotinamide and 2'-O-acetyl-ADP-ribose (OAADPr) [199]. Studies on the kinetics and biochemical properties of the enzymes revealed binding to the acetyl lysine substrate prior to NAD<sup>+</sup>. This is followed by nicotinamide cleavage from NAD<sup>+</sup>. that is the first product released, followed by deacetylated lysine and OAADPr [200]. All sirtuins are strictly NAD<sup>+</sup> dependent, a distinct characteristic that distinguishes them from other deacetylases. In fact, SIRT6 is a sirtuin capable of tightly binding to NAD<sup>+</sup> without the requirement of an acetylated substrate, indicating that it may function as a NAD<sup>+</sup> sensor [201].

Besides being an endogenous product of the deacetylation reaction, nicotinamide is also a well-known inhibitor of sirtuins. Nicotinamide is an amide of nicotinic acid (vitamin B3) and is part of common enzyme co-factors like NAD<sup>+</sup> (nicotinamide adenine dinucleotide) and NADP (nicotinamide adenine dinucleotide phosphate) [202]. Intracellular physiological levels of nicotinamide in some mammalian cells seem to be in the range similar to the IC<sub>50</sub>'s of some sirtuins reinforcing the hypothesis that some sirtuins may act as NAD<sup>+</sup> and nicotinamide sensors [203, 204].

O-acetyl-ADP-ribose is another product of the deacetylation reaction [205]. Early studies characterizing this molecule found that quantitative microinjection into starfish oocytes led to a blockage of oocyte maturation, indicating for the first time that OAADPr can evoke a biological activity [206]. There is now mounting evidence that OAADPr can elicit downstream responses that might synergize or antagonize the biological functions of sirtuin genes. So far, OAADPr has demonstrated to be related with functions in gene silencing, ion channel modulation and cell redox state maintenance [205].

### 3.2.2. ADP-ribosylation activity

Another reaction catalyzed by sirtuins is ADP-ribosylation. Although sirtuins were firstly described as ADP-ribosyltransferases, their deacetylase activity has quickly

overshadowed this activity, and as a consequence the biological processes associated to this reaction remain poorly understood [207]. In truth, sirtuins may have just deacetylase activity, both, or be mostly ADP-ribosyltransferases. An example is SIRT4 that is an efficient *in vitro* ADP-ribosyltransferase of histones, that only recently was discovered to possess deacetylase activity [208, 209]. There is an active debate on whether ADP-ribosylation is in fact a biologically relevant function of sirtuins, or just an irrelevant side reaction/non-enzymatic artifact [210]. Notwithstanding, some of the players in the dynamics of intracellular ADP-ribosylation have only been recently identified and it is becoming apparent that this PTM might be relevant for the modulation important cell processes and signaling pathways like signal transduction mechanisms, transcription and DNA repair [211].

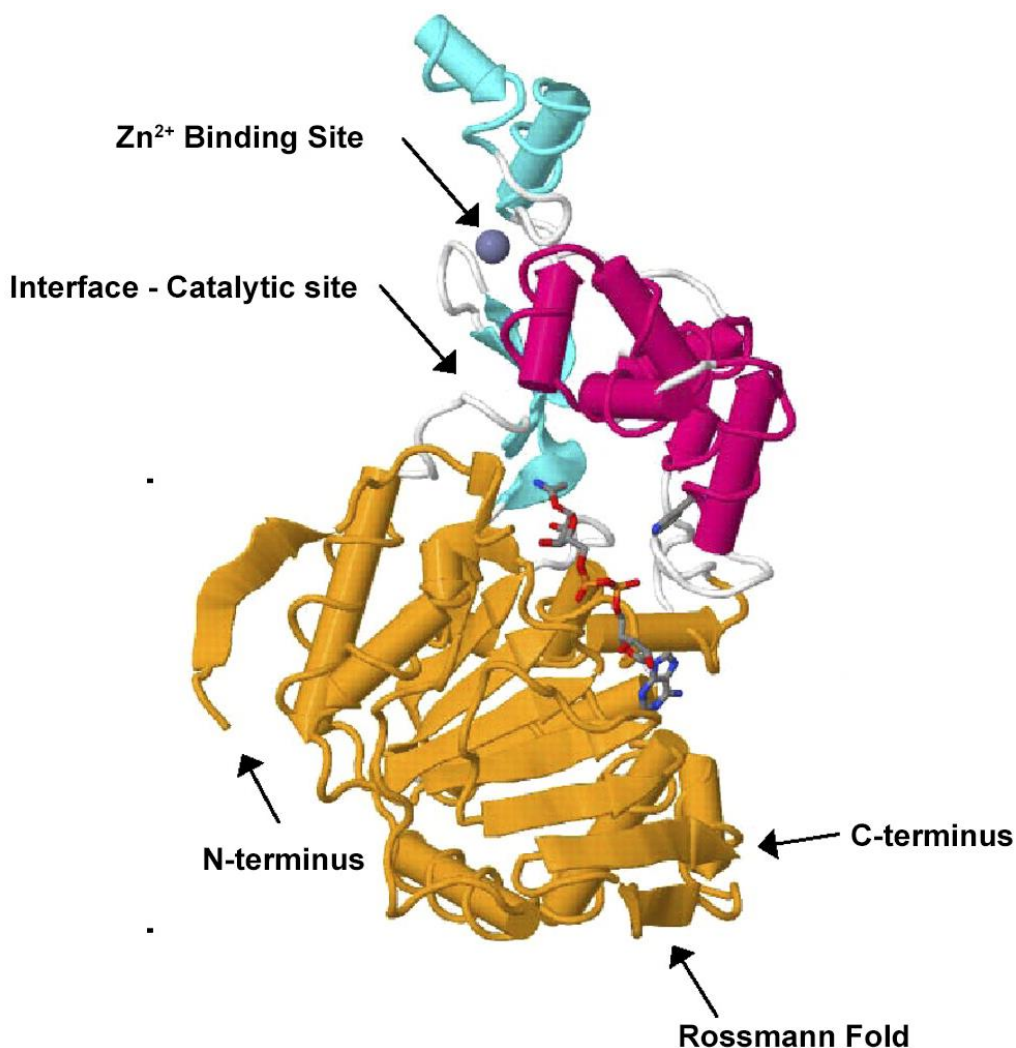
### 3.2.3. Other functions

Although (de)acetylation is the most common PTM (de)acylation, other groups can be targeted, like succinyl and malonyl groups. SIRT5 and SIRT6 are some examples of proteins that perform deacylations of lysines other than acetyl groups, and their activities are important regulators of cell functions [212-214].

### 3.3. Structural features

There is a wealth of information regarding the structural features of sirtuins. To date, some 83 structures of sirtuins are available in the protein databank, many of them co-crystallized with natural ligands or inhibitors. Though the available structures range from bacterial to mammalian sirtuins, the majority of the structures originate from the human genome.

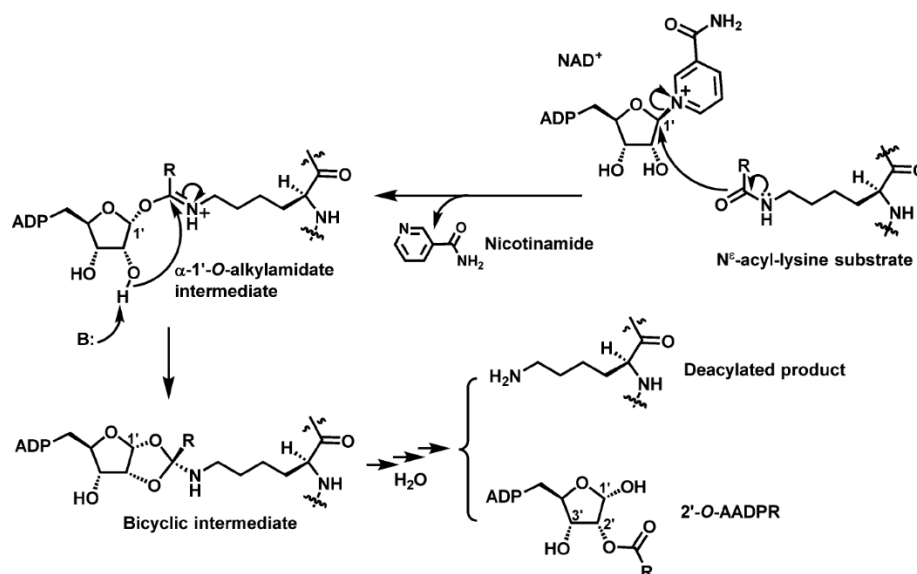
Although the sequence homology can vary significantly between sirtuins, especially between prokaryotic and eukaryotic proteins, there is a conserved catalytic core of about 250 amino acids common to all members in the family [215]. The structure similarity of PfSir2A with mammalian SIRT5, despite a sequence homology of just 33% is a clear illustration [215]. This core contains a Rossmann fold domain that is a NAD<sup>+</sup> binding site, and a Zn<sup>2+</sup> binding domain containing four highly conserved cysteine residues arranged in the motif (CX<sub>2</sub>CX<sub>20</sub>CX<sub>2</sub>). The catalytic site is located inside a hydrophobic channel formed at the interface of this two domains [215] (Figure 7).



**Figure 7.** Ribbon representation of the yeast Sir2 in complex with an acetyl-ribosyl-ADP-intermediate. The zinc binding site is represented in light blue and the NAD<sup>+</sup>-binding Rossman fold in orange. [Adapted from [216]].

Whereas in HDACs from class I, II and IV Zn<sup>2+</sup> is an active participant in catalysis by producing free acetate and deacetylated lysine, in sirtuins it does not participate in reaction. However, the metal is essential for structural integrity, as was elucidated by the reversible loss of activity in a *P. falciparum* sirtuin where the zinc ion was removed [217]. Interestingly, an exception to the conservation of the CX<sub>2</sub>CX<sub>20</sub>CX<sub>2</sub> motif is found on some sirtuins of trypanosomatids, where one of the cysteines is not present [218]. However, deacetylase activity does not seem to be affected [219]. The molecular mechanism of deacetylation is still not completely elucidated, but it is generally accepted that the first step in the reaction involves the nucleophilic addition of the acetyl oxygen to nicotinamide ribose by a mechanism of S<sub>N</sub>2 substitution to produce O-alkylamine intermediate and nicotinamide. Then the acetyl group is transferred to ADP-ribose to form O-acetyl-ADP-ribose and deacetylated lysine [220] (Figure 8).





**Figure 8.** Molecular mechanism of sirtuin NAD<sup>+</sup>-dependent deacetylation. [Adapted from [221]].

### 3.4. Known members of the sirtuin family

#### 3.4.1. Yeast Sir2

The founding member of the sirtuin family is Sir2 from the budding yeast and was initially identified as part of a protein complex necessary to silence the expression of the mating-type-loci [222, 223]. Subsequently, it was also implicated in transcriptional silencing at telomere proximal sites [224] and ribosomal repeats at the ribosomal DNA (rDNA) locus [225-227]. Sir2 can be associated in distinct protein complexes that vary according to target site. For instance, at telomeres and the mating-type-loci, Sir2 forms a complex with two other homologues, Sir3 and Sir4 [224], while at rDNA locus Sir2 associates with Net1 and Cdc14 to form the regulator of nucleolar silencing and telophase exit - RENT complex [228, 229]. Yeast cells lacking Sir2 present a reduced lifespan that has been correlated with the accumulation of extrachromosomal ribosomal DNA circles originating from illegitimate recombination that are toxic to the cell and have been associated with aging [227, 230, 231].

#### 3.4.2. Mammalian sirtuins

SIRT1 is the most extensively studied member among the sirtuin family. The TATA box-binding protein-associated factor RNA polymerase I subunit B (TAF<sub>I</sub>68) was the first

substrate to be identified for SIRT1 in mouse cells. It is a transcription factor necessary for regulating the RNA polymerase I transcriptional complex, where it was shown that deacetylation inhibits transcriptional initiation *in vitro* [232]. Studies on p53, a non histone substrate, demonstrated that acetylation activates the DNA-binding activity and target gene expression, also increasing its stability [233]. Consistent with this proposed SIRT1 inhibition of p53 function, SIRT1 knockout mice exhibit p53 hyper-acetylation and increased radiation-induced apoptosis, suggesting that SIRT1 can facilitate tumor growth by antagonism of p53 [234]. Still, the fact that SIRT1 can be found either overexpressed or underexpressed in different tumor types, and the finding that it can also function as a tumor suppressor [235] has hindered the clarification of its role in tumorigenesis. SIRT1 also plays an important role in metabolism, and its relation with caloric restriction and life-span extension has received much attention (reviewed in [236-238]). The beneficial effects of caloric restriction have been focused on the insulin-like growth factor-1 (IGF-1) and the target of rapamycin (TOR) pathways [239, 240], but increasing evidence suggest a role of SIRT1 in caloric restriction in mammals as well. For instance, SIRT1 expression was found to be elevated in models of caloric restriction, like fasting mice, low calorie diet in rat, or humans on a 25% caloric restriction diet [241-243]. On the other hand, mice lacking SIRT1 lost the life-span extension benefits of a 40% reduced calorie diet [244]. Despite many studies, the exact molecular mechanisms of SIRT1 in caloric restriction are still to be unraveled.

*In vitro* studies attribute a role to human SIRT2 in cell cycle regulation through the deacetylation of both tubulin and histone H4 [193, 245, 246]. In particular, it has been found that SIRT2 overexpressing cells were significantly delayed in cell cycle progression through mitosis [247]. Some links with age-related diseases have been described for SIRT2, such as neurodegenerative diseases [248-250], as well as cancer. Mice lacking SIRT2 are prone to the appearance of tumors, thought to be mediated by the negative regulation of the anaphase-promoting complex by SIRT2 [251]. SIRT2 has been demonstrated to have decreased expression in human gliomas, some of the most frequent malignant tumors in the brain [193, 252]. Indeed, the SIRT2 gene is located at a chromosomal region frequently deleted in human gliomas. In addition, it was found to be an effective demyristoylase with a catalytic efficiency slightly higher for myristoyl groups than acetyl groups, although no physiologic target or biological relevance of this acyl modification has been described yet [253].

SIRT3 is the major mitochondrial deacetylase and studies with double knockout mice have revealed high levels of acetylation in protein targets [254, 255]. In addition, it was observed that these mice have impaired production of ATP [254]. When fasting or fed a high-fat diet, the mice display atypical phenotypes that include cold intolerance and

decreased ketone body formation [256, 257]. This strengthened the link with thermogenesis that had been previously demonstrated [258, 259]. In fact, SIRT3 expression is induced in mice in both white and brown adipose tissue upon caloric restriction and exposure of brown adipose tissue to cold temperatures [260]. In addition, SIRT3 also has a role in the deacetylation and activation of fatty acid oxidation, amino acid metabolism, electron transport chain and antioxidant defenses [261, 262].

SIRT4 was originally thought to be an unusual sirtuin due to the lack of deacetylase activity [263]. However, it was shown to ADP-ribosylate and down-regulate glutamate dehydrogenase production of ATP and has been implicated in insulin regulation of  $\beta$ -cells [208, 263]. Moreover, SIRT4 has recently been attributed a tumor suppressive function due to its involvement in DNA damage protection mediated by inhibition of mitochondrial glutamine metabolism, suggesting it might have therapeutic potential for treating glutamine-dependent cancers [264]. This mechanism has been shown to be repressed by mammalian target of rapamycin complex 1 (mTORC1) pathway [265]. SIRT4 also coordinates the balance between lipid synthesis and their catabolism by repressing malonyl-CoA decarboxylase, proving that it has, in fact, deacetylase activity [209].

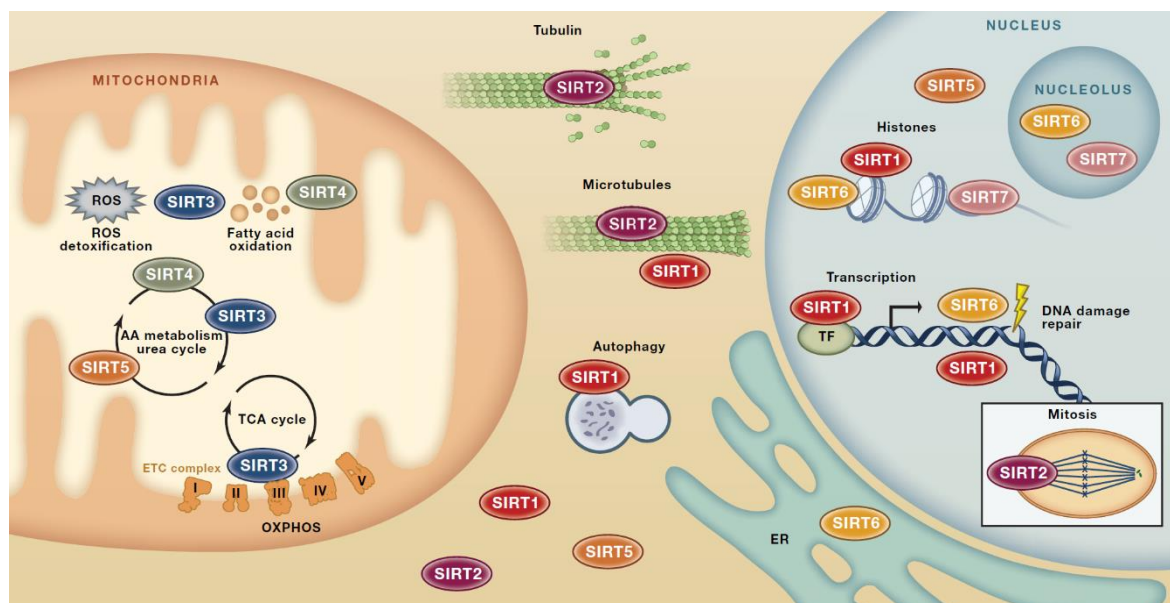
SIRT5 is a NAD<sup>+</sup>-dependent protein lysine demalonylase and desuccinylase [266] and also has a deglutarylase activity [267]. It has a deacetylase activity [191], but has preference for acylcarboxyl negatively charged groups [266, 267]. Some of its functions are related to glycolysis modulation [214]. The succinylome of mammalian cells has revealed many points of succinylation that are probable targets of SIRT5, mostly concentrated on mitochondrial metabolism [213]. SIRT5 also promotes urea cycle function via the regulation of carbamoyl phosphate synthase [191, 196], and purine metabolism via urate oxidase [268]. Although a global protein hypersuccinylation and elevated serum ammonia during fasting were observed in SIRT5 knockout mouse model, the enzyme deficiency did not lead to any major metabolic abnormalities under either low or high fat diet conditions. These observations suggest that SIRT5 is likely dispensable metabolic homeostasis under the basal conditions. It remains to be evaluated the role of SIRT5 in extreme conditions [269].

While most mammalian sirtuins have been mostly implicated with metabolism, SIRT6 seems to be the only one with a direct link supporting a defined role in mammalian aging [270]. In fact, mice lacking SIRT6 gene develop a progeroid-like symptom with loss of subcutaneous fat, curved spine and lymphopenia. They develop normally for 2 weeks after birth, but then suffer from acute degeneration processes, ending up dying at 1 month of age [271]. At first considered to not possess deacetylase activity, but solely an ADP-ribosyltransferase activity [272], it was later found that SIRT6 removes both acetyl and long chain fatty acyl groups from target molecules [212, 273]. It is localized in the nucleus,

associated with chromatin, where it promotes the specific NAD<sup>+</sup>-dependent deacetylation of H3K9 and H3K56 [273-275]. SIRT6 is involved in genome protection by assuring correct telomere maintenance [273, 274], as well as DNA repair by double-strand break repair machinery [276, 277]. Like other sirtuins it also has a role in metabolism by influencing both glycolysis and gluconeogenesis [278-280] and lipid metabolism by regulating triglyceride synthesis [270, 281]. Conditions like inflammation, heart disease and cancer all seem to be linked with SIRT6 function [282].

SIRT7 is the least studied sirtuin of all the mammalian sirtuins, but recent findings have established new functions and roles for this protein. It is a nucleolar sirtuin [283] and its localization is associated with the main process happening at this sub-nuclear structure, namely rDNA transcription [284]. SIRT7 does not possess a very strong deacetylase activity towards common synthetic and natural peptides [283], which is in agreement with the fact that SIRT7 depletion in mice did not change the global acetylation levels of either nucleus or nucleolus proteome [285], indicating that SIRT7 deacetylase activity is specific to a limited set of proteins. One example is specific deacetylation of H3K18 [192, 286] that underlies its role in chromatin remodeling. Also, SIRT7 has been found to be closely associated with B-WICH complex, a chromatin remodeling complex [287]. It also has a role in protein synthesis [285, 288] and contributes to cell survival, namely by protecting against genomic insult [289, 290], hypoxia [291] and low glucose induced stress [292]. All the functions described characterize SIRT7 as a pro-survival protein. Indeed, it is currently considered to be an oncogene in all the cancer types studied so far [288, 293, 294].

A schematic representation of the localization and major biologic functions of mammalian sirtuins is represented in Figure 9.



**Figure 9.** Representation of the seven mammalian sirtuins regarding their sub-cellular localization and attributed biological roles. [Adapted from [295]].

### 3.4.3. Protozoan sirtuins

#### 3.4.3.1. *Plasmodium* spp.

Parasites of *Plasmodium* spp. have been characterized with two sirtuin orthologues, called Sir2A and Sir2B. Sir2A is the most extensively studied homologue, mainly located at the nucleus [296] although it can also shuttle to cytoplasm after posttranslational SUMOylation [297]. Sir2A has been characterized as a mediator of transcriptional silencing at the telomeric regions of chromosomes [296, 298]. The telomeres of *P. falciparum* are rich in gene families involved in antigenic variation such as the *var* family of genes. These genes are responsible for the expression of parasite-derived *P. falciparum* erythrocyte membrane protein, PfEMP1, responsible for immune evasion in humans [299]. The family of *var* genes is tightly regulated by sirtuins, with the expression of its members being mutually exclusive [300, 301]. The switch of active *var* is controlled exclusively at the epigenetic level [300, 302].

PfSir2B is a larger homologue with a molecular weight more than four times the size of Sir2A and is involved in the transcriptional silencing of a complementary subset of *var* genes with distinct promoter types [303].

### 3.4.3.2. *Leishmania* spp.

Sirtuins from *Leishmania* spp. parasites were among the first to be identified in trypanosomatids, when a complementary DNA (cDNA) isolated and sequenced from *Leishmania major* showed a high homology with yeast Sir2 [304]. Antibodies raised against this LmSir2 later showed to be reactive against different life cycle stages of *Leishmania major*, but also of *Leishmania amazonensis* and even the serum of a patient infected with *Leishmania infantum* [305]. Furthermore, the protein was found to be among the secreted material of *L. major* [305].

Overexpression of the orthologous protein in *L. infantum*, sharing 93% homology to the *L. major* protein, led to an increased survival of amastigotes under axenic conditions [306]. Also, when the overexpression was performed in mammalian fibroblasts, host cells became more permissive to infection by *Leishmania* infection in comparison with wild type cells, hinting at a modulation of host cell by the parasite [307]. Genetic knockouts in *L. infantum* Sir2rp1 gene also contributed the elucidation of the importance of this protein in the parasite. While single knockouts were readily obtainable, double deletion of the alleles was only possible after the rescue by an ectopical copy of the gene, suggesting an essential role for parasite survival [308]. When single-knockouts of *L. infantum* Sir2rp1 were used to infect a macrophage cell line, *in vitro*, it was noted that although they had the same invasive capacity than wild-type parasites, they showed a hindered replication rate leading to diminished infections over-time. Furthermore, the mutant parasite also failed to establish infection in an *in vivo* mouse model of leishmaniasis [308]. Cellular and biochemical studies later established LiSir2rp1 as NAD<sup>+</sup>-dependent deacetylase with ADP-ribosylation activity that co-localized to the cytoskeleton and potentially interacted with tubulin as well as with HSP83, an orthologue of mammalian HSP90 [309, 310]. The association with cytoskeleton is a characteristic feature of both SIRT2 and HDAC6 [193, 311]. In addition, an orthologous from *L. amazonensis*, LaSir2rp1 was found to be a glycosylated protein, but whether this is the case for other species remains to be seen [312]. Although the Sir2 related protein 1 has received much attention, no studies have been made for the other two proteins codified by the *Leishmania* species, rp2 and rp3.

Recently, Sir2rp1 from *L. donovani* has been implicated in the resistance of amphotericin B, a reference drug in the treatment of visceral leishmaniasis. When clinical isolates were targeted for gene knockout of the protein, parasites showed a lower level of multi-drug resistant transporter MDR1, lower drug efflux, increased ROS production and increased sensitivity to amphotericin B [313]. On the contrary, overexpression led to a resistant phenotype, thereby suggesting Sir2 as a new resistant marker for visceral

leishmaniasis [313]. Comparative transcriptomic analysis also implicates Sir2 in resistance to miltefosine, another drug used to treat the disease [314].

In addition to its potential as a novel drug target, Sir2rp1 from *Leishmania* spp. has also been suggested as a vaccine [315, 316].

### 3.4.3.3. *Trypanosoma brucei*

*Trypanosoma brucei*, like *Leishmania* spp., has 3 sirtuins annotated in its genome. The first enzyme to be characterized in the parasite was TbSir2rp1 [317]. The enzyme is localized mainly in the nucleus in association with chromosomes. The protein was shown to possess both deacetylase activity towards endogenous histones and also ADP-ribosylation of calf thymus histones, and to a lesser extent, bovine serum albumin (BSA). Up to that time, no ADP-ribosylation had been detected in common members of the sirtuin family like yeast Sir2 or HST2, and TbSir2rp1 was one of the first enzymes to exhibit this dual activity [318-321]. Furthermore, mutation of a catalytic histidine essential for deacetylase activity also affected ADP-ribosylation activity, suggesting that the two activities were occurring simultaneously. Because of the increased or decreased resistance to DNA damage caused by the alkylating drug methyl methanesulfonate (MMS) in overexpressing or RNAi-induced knockdown *T. brucei* cell lines, respectively, TbSir2rp1 was also attributed a role in DNA repair in this organism [317].

A later study performed in bloodstream forms (as opposed to insect stage forms in the previous work) characterized TbSir2rp1 and also the other two sirtuins, TbSir2rp2 and TbSir2rp3 that both have a mitochondrial localization [322]. TbSir2rp1 confirmed to be a nuclear protein, but when overexpressed to high levels in *T. brucei* cells, it localizes to the cytoplasm, with toxic effects to the parasite [322]. Besides, the authors performed gene knockouts for the three proteins and observed no growth defect or impairment while cultured in standard conditions [322]. TbSir2rp1 mutants, however, did show an increased sensitivity to MMS damage, confirming the previous results performed with RNA interference (RNAi). The particular localization of TbSir2rp1 led to the investigation of Sir2 mediated telomere gene silencing like the one that occurs in yeast or *Plasmodium* spp. [323, 324]. Although TbSir2rp1 confirmed to be an active player in the latter role, it was not required for antigenic variation as described for *Plasmodium* Sir2 [296, 298].

TbSir2rp1 has also been studied as a model sirtuin with both deacetylase and ADP-ribosylation activity. Biochemical studies revealed that ADP-ribosylation is 5-fold less active than the deacetylation reaction, and occurs only in the presence of an acetylated substrate

by two distinct biochemical mechanisms [325]. The latter claim has since been challenged by another research group that demonstrated that ADP-ribosylation can occur in arginine independent of the presence of an acetylated substrate, as supported by mass spectrometry and molecular dynamics simulations [326].

#### 3.4.3.4. *Trypanosoma cruzi*

Although the draft of the *T. cruzi* genome has been published a decade ago, it was not until recently that the first experimental studies involving the sirtuins of this parasite have been published [19]. Unlike *Leishmania* spp. and *T. brucei* that possess three Sir2-like proteins, *T. cruzi* only has two coding sequences annotated in its genome, TcSir2rp1 and TcSir2rp3.

One study employing parasites overexpressing TcSir2rp1 and TcSir2rp3 by a tetracycline inducible vector characterized some of the features of both proteins [218]. Localization studies employing both wild type parasites and polyclonal sera raised against both proteins, as well as localization of tagged copies in the overexpression mutants with monoclonal antibodies, attributed a cytoplasmic localization to Tcsir2rp1 and a mitochondrial to TcSir2rp3 [218]. Both of the proteins' levels are regulated throughout the life cycle of the parasite, with a significant decrease in both at the trypomastigote stage [218]. Overexpression of TcSir2rp1 was responsible for higher metacyclogenesis and higher infectivity of Vero cells. Since metacyclogenesis occurs under nutrient deprivation, it is hypothesized that TcSir2rp1 may function like sirtuins from other organisms that respond to starvation [263, 327, 328]. On the other hand, overexpression of TcSir2rp3 led to a decrease in epimastigote replication time, lower infectivity in Vero cells, increased amastigote replication and normal metacyclogenesis [218]. Because of the oxidizing environment in which amastigotes replicate, it has been suggested that TcSir2rp3 performs protecting functions against oxidative stress like SIRT3 [197]. Both of the overexpressing cell lines reduced the levels of acetylation for particular proteins as well as protected against the effect of specific sirtuin inhibitors [218].

Moretti and colleagues also independently characterized both of the sirtuins in a simultaneous study [219]. In their study, they show that salermide, a sirtuin inhibitor analogue of sirtinol found to be a strong anticancer molecule, is active against both *in vitro* cultures of epimastigotes, and against an *in vivo* model of infection by *T. cruzi*, albeit at moderate levels [219, 329]. Salermide was also found to be a strong inhibitor of TcSir2rp3 recombinant protein [219]. The authors report the same localization for both proteins, as



well as the interference in epimastigote growth, metacyclogenesis, infectivity of host cells and amastigote replication in lines overexpressing the sirtuins, though with some differences to the previous study, probably due to the amount of overexpression achieved [219].

### 3.5. Modulation of sirtuin activity

Because of the many roles sirtuins undertake in cells and the links between sirtuin deregulation and disease, active research on sirtuin modulators has been made in the search for molecules that would eventually be beneficial for human health.

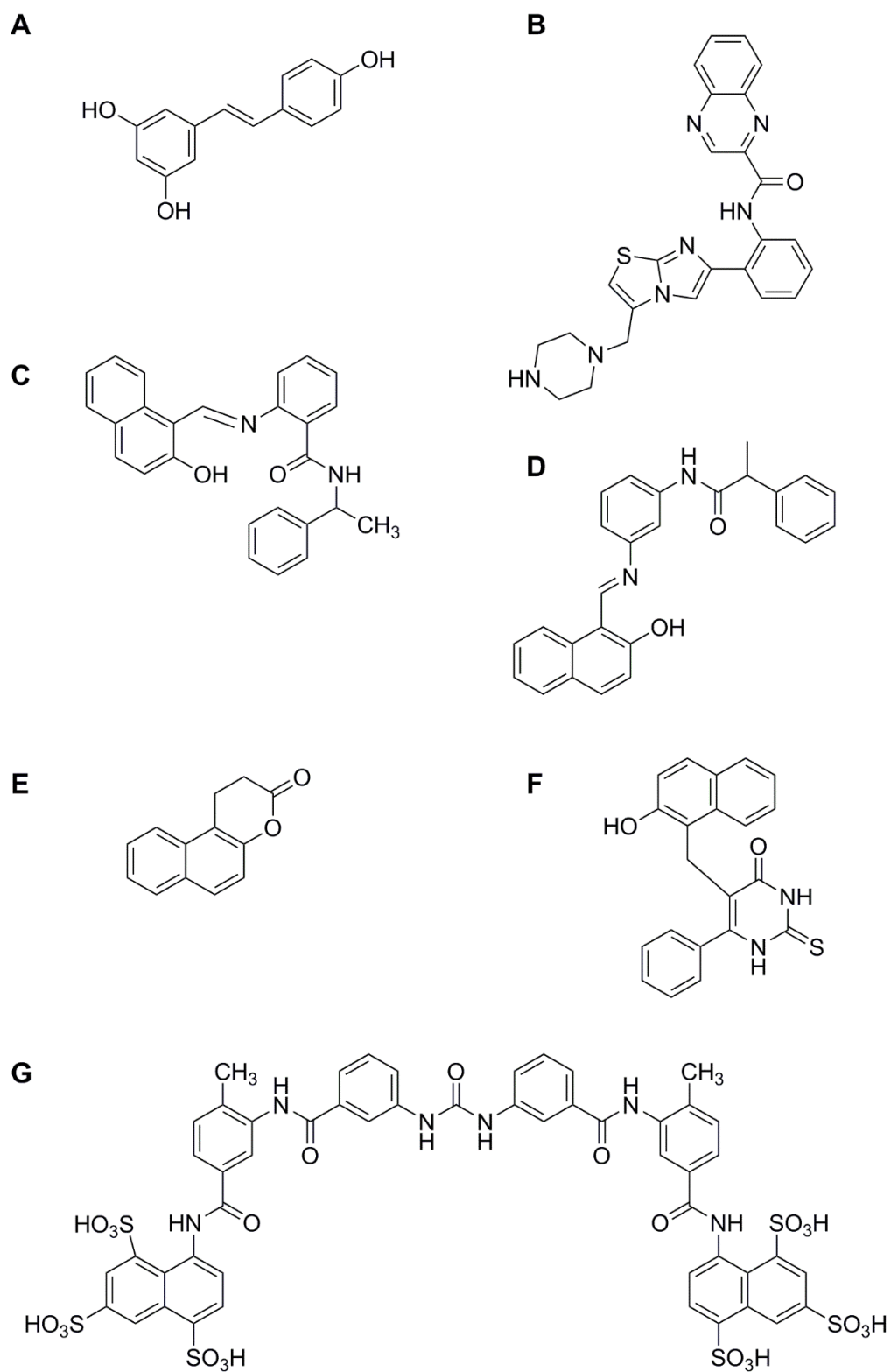
As described earlier, high SIRT1 activity has been found to be beneficial in a range of conditions. This has led to investigations in molecules that could positively increase SIRT1 function. Initially discovered to mimic caloric restriction by a mechanism of sirtuin stimulation [330, 331], resveratrol and derivatives are considered the central compounds capable of sirtuin activation [332]. This polyphenol demonstrated significant protection against neurodegeneration and has also improved general health in mice fed with a high calorie diet [333, 334]. Due to its poor bioavailability, resveratrol formulations have been developed, and tests in humans have demonstrated benefits in healthy obese men like decreasing hepatic lipid content, triglycerides and inflammation markers [335, 336]. Other molecules structurally unrelated to resveratrol like SRT1720 have also been reported to be beneficial for obesity related parameters and other conditions, but never demonstrated an increase of lifespan in animals fed without high fat diet [337-339].

High controversy has been generated involving the SIRT1 mediated mechanism of these inhibitors, as many of them seem to be only working in some types of deacetylation assays and fail to be active against endogenous protein-derived acetylated peptides [340, 341], leading to the hypothesis that other mechanisms like activation of AMPK may be taking place [342, 343].

Even though the positive modulation of SIRT1 activity favored the discovery and design of pharmacological activators of sirtuins, SIRT1 inhibitors can also be potentially useful as therapeutic agents because SIRT1 has been described to be up-regulated in cancer cell lines [235]. Furthermore, sirtuin inhibitors have also been proposed for the treatment of other age-related human diseases like Parkinson's disease [250], and infectious diseases such as leishmaniasis [344], and HIV [345], and others. Additionally, SIRT3, SIRT4, and SIRT5 are located into the mitochondria, where they mediate

deacetylation of proteins that participate in processes important for energy metabolism and apoptosis that may be implicated in diseases like cancer and metabolic syndrome [346].

Sirtinol was discovered by phenotypic screens to be an inhibitor of both yeast Sir2 and SIRT2, and has some anticancer potential in breast and lung cancer [347, 348]. Salermide is an analogue of sirtinol with a stronger *in vitro* inhibitory effect towards SIRT1 and SIRT2 enzymes, and it is a strong inducer of p53-independent apoptosis in cancer cells [329]. Other inhibitors include suramin, an inhibitor with several biologic activities, including antitrypanosomal [349], although not by a mechanism of sirtuin inhibition; cambinol, a sirtinol analogue with proved antitumoral *in vivo* efficacy [349]; and splitomycin [350].



**Figure 10.** Chemical structures of sirtuin modulators: **A)** Resveratrol; **B)** SRT1720; **C)** Sirtinol; **D)** Salermide; **E)** Sambinol; **F)** Splitomycin; **G)** Suramin.



# **PART IV**

## Objectives and results



#### 4.1. Scope of the thesis

Human trypanosomiasis constitutes one of the most neglected group of diseases in the world, affecting especially poor people in the African and American continent. Chagas disease in particular is caused by the protozoan parasite *Trypanosoma cruzi* and is responsible for significant mortality and morbidity in Latin America [351]. Disease control has relied heavily on vectorial eradication programs, but the high number of new and past cases of infection have left an estimate of 6 million people worldwide infected with *T. cruzi* [66]. Treatment for Chagas disease is limited to the use of chemotherapeutic agents that are decades old and have problems such as safety, efficacy and the need for long administration periods [352]. Some clinical trials launched in the last decade constitute a progress in drug development for the disease, but have, unfortunately, failed to deliver a new therapeutic [173, 353]. The need for alternative drugs is therefore an urgent need. The first steps in drug discovery start with screens for active small molecule inhibitors of either the whole parasite (phenotypic strategy) or a biochemical defined and purified molecular target (target-based strategy) [354]. The experimental work supporting this thesis employed both of the approaches in the search for new starting points that could be used in trypanosomiasis drug discovery. In the first part of the results section, the phenotypic approach is introduced by the development and validation of high-content screening (HCS) assays successfully employed in the discovery of active compounds originating from a targeted library of kinase/phosphatase-like inhibitors (section 4.2.1.1). Furthermore, the application and versatility of the technology is demonstrated in the evaluation of the enantiomers of nifurtimox against several *T. cruzi* lineages (4.2.1.2) and in the characterization of the antiparasitic activity of two families of synthesized compounds: naphthalimides (4.2.1.3) and 7-aryltioetherthieno[3,2-b]pyridines (4.2.1.4). The second part exploits the potential of Sir2rp1 inhibitors as novel drugs against *T. cruzi* (4.2.2.1) and *T. brucei* (4.2.2.2).

In particular, the objectives of this thesis were:

- Validation of a phenotypic HCS assay for *T. cruzi* with a focused library of kinase/phosphatase-like small molecule inhibitors;
- Development of a phenotypic profiling assay capable of identifying compounds that interfere with the intracellular stages of *T. cruzi* life cycle;
- Evaluate the potential of Sir2rp1 as a drug target in *T. cruzi*;
- Evaluate the potential of BNIP derivatives against Sir2rp1 enzymes and whole parasites of *T. brucei* and *T. cruzi*.





## **4.2. Results**

### **4.2.1. Phenotypic drug discovery**

#### **4.2.1.1. High-content screening assays for Chagas disease drug discovery**

Carolina B. Moraes, Luís Gaspar, Adalberto M. Araujo-Junior, Jair L. Siqueira-Neto, Dawoon Han, Nakyoung Lee, Anabela Cordeiro-da-Silva, Sergio Schenkman and Lucio Freitas-Junior.

*Manuscript in preparation*

#### **Objectives of the study:**

The present study aimed at the development of a high-content drug screening assay for *T. cruzi*. The use of the clinically relevant amastigote stage, the adaptation to high throughput, the reproducibility and the use of non-genetically modified parasites and host cells were the main objectives. Furthermore, a phenotypic assay that allows the visualization of the life cycle stage being affected by drugs was also developed and used to characterize compounds that stopped parasite development at distinct points.

#### **Main results:**

→ A high-content drug screening assay against *T. cruzi* with a high statistical validity was established.

→ Of a total of 4,000 compounds screened, 119 hits were confirmed to be active against the parasite, as assayed by dose response curve analysis. Furthermore, 11 compounds were at least 5 times more selective towards *T. cruzi* parasites than host cells.

→ A secondary analysis revealed an additional subset of compounds that were able to interfere with the development of the intracellular life cycle stages of the parasite. With the aid of a newly developed high-content immunofluorescence that marks amastigotes and

trypomastigotes of *T. cruzi*, it was possible to determine the stage of differentiation being affected by the drugs.

### **Conclusion:**

The present study reports the development of two HCS assays that could have a potential use in drug discovery for *T. cruzi*. The use of unmodified cells, the adaptability to 384-well plates and the high statistic value of the assay represent significant advancements in the field of drug discovery for the parasite. In addition to classic drug discovery, the immunofluorescence assay here reported should present a valuable tool when evaluating the mechanism of action of approved and newly developed drugs. The nature of the library used (kinase/phosphatase-like inhibitors) highlight the possibility of exploiting trypanosomal kinases as new drug targets against Chagas disease.

## High-content screening assays for Chagas disease drug discovery

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## ABSTRACT

Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi* and remains one of the most neglected tropical illnesses in the world. It is endemic to Latin American countries and is considered to be the parasitic infirmity with the biggest local social and economic burden. Benznidazole and nifurtimox, the only drugs available to treat the disease, were introduced more than four decades ago, with undesirable side effects and low efficacy on the chronic phase of infection. Although some drugs have entered clinical trials in recent years, no major approval added new treatment options for Chagas disease, maintaining the pressure for the discovery of new drugs.

In this work we report a HCS of 4,000 kinase inhibitor-like compounds using an *in vitro* model of infection of U2OS cells with Y strain *T. cruzi* trypomastigotes. A primary analysis of the results focused on the identification of active compounds able to reduce the ratio of infected cells, with a custom software developed in-house. A secondary analysis aimed at identifying molecules that interfere with the parasite intracellular development by marking amastigotes and trypomastigotes with specific antibodies.

## AUTHOR'S SUMMARY

*Trypanosoma cruzi* is a protozoan parasite belonging to the Kinetoplastida class responsible for Chagas disease, a neglected tropical illness that affects an estimated 6 to 8 million people in Latin America, with another 25 million in risk of acquiring the disease and a death toll of 12,000 every year. Commonly transmitted with the bite of the triatomine bugs, the disease is characterized by a nearly asymptotically acute phase but a problematic chronic phase in which 20-30% of individuals develop serious cardiac and/or intestinal problems. The therapies actually used to treat the disease have been introduced more than forty years ago, have adverse effects and are mainly active in the acute phase. There is, therefore, an urgency to find better alternatives. The screening of small molecule libraries is a central starting point to the discovery of active compounds against the parasite.

Here we report an image-based HCS of 4,000 compounds from an anti-kinase-like library using the Y strain of the parasite invading U2OS cells. From this screening, we discovered 2 promising clusters able to clear the parasite from infected cells with low toxicity as well as compounds able to interfere with the normal intracellular development of the microorganism.

## INTRODUCTION

Discovered more than 100 years ago, Chagas disease, also known as American trypanosomiasis, remains one of the most neglected tropical diseases in the world. It affects mostly the South and Central American continent, with estimates of 6-8 million cases, other 70 million people in risk of acquiring the disease, and about 12,500 deaths every [1,2]. The etiologic agent is the protozoan parasite *Trypanosoma cruzi*, a kinetoplastid with a complex life cycle. The human transmission happens with the bite of an infected hematophagous triatomine insect vector that releases contaminated feces in the proximity of the skin lesion [3]. The infective form metacyclic trypomastigotes easily infects local cells and inside the cell changes into the replicative form amastigote. After 5 to 6 days, the amastigotes differentiate into bloodstream trypomastigotes that burst the host cell and migrate to other parts of the body through the blood and lymph, where they can freely invade other cells or be ingested by an insect vector. In the triatomine gut the parasite transforms once again into an insect-specific replicative stage called epimastigotes and after several divisions it migrates to the final portion of the intestine where it differentiates once again into metacyclic trypomastigotes, thus closing the cycle [4].

The vectorial transmission is still responsible for some 41,000 cases every year, but infection with the parasite can also happen through other routes such as transfusions [5], congenitally [6] and orally [7]. These transmission mechanisms coupled with migration flows have, in the past years, contributed to a worldwide spread of the disease out of its endemic zones, making it a global concern [8]. After infection, an acute phase follows, and is most of the times asymptomatic or presents unspecific signs for 4 to 8 weeks. Unless treated, the parasite remains in the host for his lifetime, but only 30-40% of the individuals will present the chronic phase of the disease, characterized by cardiac symptoms, gastrointestinal alterations, or both, usually 10 to 30 years after the first infection [9]. Benznidazole and nifurtimox, two drugs developed more than forty years ago, are the only treatments available. Severe side effects, lack of efficacy in the chronic phase and the long periods of treatment necessary to achieve cure make these drugs far from ideal [10]. The lack of alternatives has prompted an increase in the number of drugs entering clinical trials for Chagas disease in the last years, most notably the ergosterol biosynthesis inhibitor posaconazole and K777, a cysteine protease inhibitor that targets cruzain, the major proteolytic enzyme in *T. cruzi* [11]. There is, however, still a need for innovative drugs.

Since the sequencing of *T. cruzi* genome, many putative targets that may be exploited to treat the disease have been suggested, with the parasite kinome attracting particular interest [12]. It is composed of 190 protein kinases, a number relatively high when

compared to other intracellular protozoan parasites that transit diverse environments such as *Plasmodium falciparum*, hinting at an important role of such proteins in the parasite life cycle [13]. Kinases have been associated with parasite morphology, proliferation, differentiation and overall viability [14-16].

This paper describes the first HCS against *T. cruzi* using a targeted library of 4 000 anti-kinase-like compounds and using a dual approach to the result analysis: a classic approach that identifies and characterizes compounds able to kill the parasite and clear the infection from host cells and a phenotypic analysis aiming at identifying compounds that interfere with the parasite intracellular development.

## MATERIAL AND METHODS

### Cells and parasites

U2OS and LLC-MK2 cells (ATCC, Manassas, VA) were cultured in DMEM high-glucose medium (Wellgene LM001-05) supplemented with 10% fetal bovine serum (FBS, Gibco 16000), 100 U penicillin/mL and 100 µg/mL streptomycin (Gibco 15140) in a humid atmosphere of 5% CO<sub>2</sub> at 37°C, with sub-cultures every 2-3 days or 3-5 days, respectively. U2OS are human osteosarcoma cells readily infected by *T. cruzi* and were used to screen the chemical library. They present a large cytoplasm that facilitates parasite detection and grow in monolayers, spread from each other, crucial features for software analysis. LLC-MK2 are monkey kidney epithelial cells used to maintain *in vitro* culture of tissue culture-derived trypomastigotes (TCTs) of *T. cruzi* Y strain. Parasites were collected from the supernatant of infected LLC-MK2 cultures maintained in DMEM low-glucose (Wellgene LM001-11) containing 2% FBS and 1% penicillin/streptomycin, five or six days after infection. Y strain belongs to the *T. cruzi* sub-group II and is considered ideal for primary screening [17,18].

### Reference drug and compound library

The anti-trypanocidal reference drug benznidazole was kindly provided by Epichem (EPL-BS63) and was dissolved in DMSO at a stock concentration of either 80 or 40 mM. An anti-kinase compound library of 4000 molecules from BioFocus at a stock concentration of 10 mM in DMSO was reformatted to 384-well plates at a concentration of 5 mM in DMSO and tested in singlet.

### High-content screening

384-well black sterile plates with clear bottom wells were plated with 9.4 µL of DMEM low-glucose media and 0.6 µL of compound from the library with a CyBi-Well 384-channel pipettor (CyBio AG) for a final concentration of 20 µM for compounds and 400 µM for reference drug (EC<sub>100</sub>). Trypsinized U2OS cells at a cell density of 2x10<sup>5</sup> cells/mL were infected with a parasite suspension at 1.2x10<sup>7</sup> parasites/mL in a ratio of 1:4 (vol/vol) corresponding to 15 parasites/cell. 50 µL/well of this suspension was then added to the 384-well plates containing the compound with a WellMate (Thermo Scientific) liquid handler and the plates were centrifuged for 1 minute at 1000 rpm and incubated for 48 hours at 37°C, 5% CO<sub>2</sub>. Confirmation of active hits was done with the compounds diluted in a two-fold scheme for 10 concentrations starting at 40 µM.



### **Fixation, nuclei staining and fluorescence microscopy**

After the incubation, all wells were treated with paraformaldehyde (final concentration 4%) in PBS (Wellgene LB001-02) and fixed for 15 minutes, after which the wells were aspirated with an EL406 microplate washer-dispenser (BioTek). The staining of cell nuclei and parasites was done with 5  $\mu$ M Draq5 (Biostatus DR50200) paraformaldehyde 4% in PBS for 2 hours and then washed 2 times with 50  $\mu$ L/well of PBS. Four fluorescent images per well were acquired using an Operetta confocal automated microscope (PerkinElmer) with a 635 nm excitation filter and 20x lens magnification.

### **Immunofluorescence assay**

for the selected phenotypic hits, a high-content immunofluorescence assay was developed with some differences to the screening protocol. DMSO concentration was lowered to 0.5%. Cells were seeded one day before compound addition and infection with parasite in order to mimic a more physiologic condition and at a lower dilution ( $5 \times 10^4$ /mL) so the 72 hours time-point would not be overgrown with cells. Fixation was done similarly to the screening protocol followed by washing with PBS 3 times. Triton X-100 0.2% was used to permeabilize cells and a solution of 4% BSA (Sigma A3059) in PBS was used as blocking agent. mAb 2C2 (anti ssp-4) [19] and mAb 39 [20] (anti-trans-sialidase (TS), kindly provided by Sergio Schenkman) at 1:500 dilution were used as primary antibodies targeted at the amastigote and trypomastigotes stage, respectively. AlexaFluor® 488 donkey anti-mouse (Invitrogen A-21202, 1:200 dilution) as a secondary antibody and Draq5 were incubated for 30 minutes at room temperature and washed 3 times with PBS between additions. Seven images per well were acquired with the microscope described above.

### **Software development for image analysis**

A custom software for the analysis of the images was developed and is published in the following reference: [21].

### **Data normalization and assay quality control**

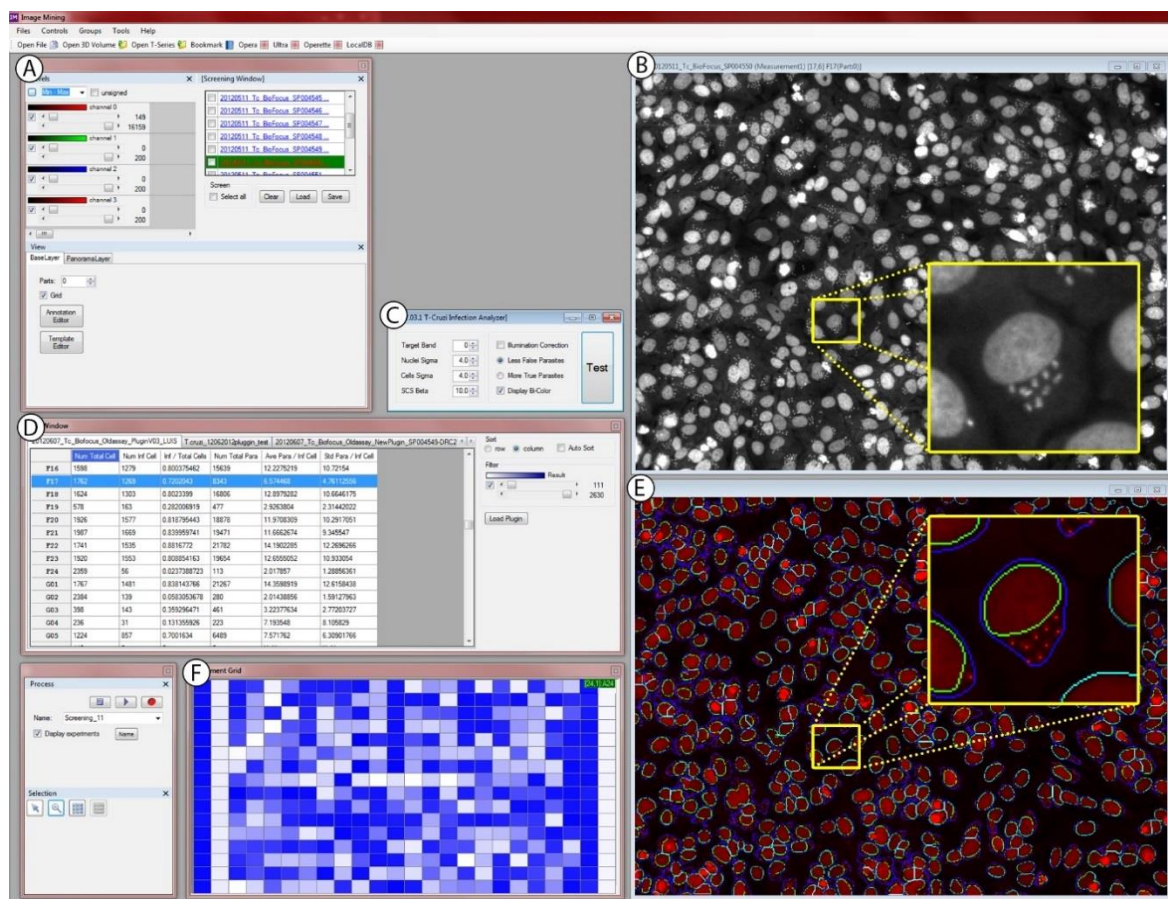
The activity was calculated by normalization of the infection ratio (number of infected cells/total cells) values obtained by the software based on the controls benznidazole ( $EC_{100}$ , effective concentration showing 100% activity, meaning minimum measured infection ratio) and 1% DMSO (vol/vol) (0% activity, meaning maximum measured infection ratio) following the formula:  $\% \text{ infection} = (\text{measured IR} - \mu_{\text{BNZ } EC_{100}}) / (\mu_{1\% \text{ DMSO}} - \mu_{\text{BNZ } EC_{100}}) \times 100$  in which  $\mu_{\text{BNZ } EC_{100}}$  is the average infection ratio of the benznidazole  $EC_{100}$  control and  $\mu_{1\% \text{ DMSO}}$  is the averaged infection ratio of all 1% DMSO control wells. The Z' value, defined by the formula

$Z' = 1 - [(3\sigma_{\text{BNZ EC100}} + 3\sigma_{1\% \text{ DMSO}})/(\mu_{\text{BNZ EC100}} - \mu_{1\% \text{ DMSO}})]$  where  $\sigma_{\text{BNZ EC100}}$  and  $\sigma_{1\% \text{ DMSO}}$  are the standard deviations of both controls, is used to quantify the suitability of a particular high-throughput/content screening as a measure of statistical effect size [22].  $\text{IC}_{50}$  (inhibitory concentration) and  $\text{CC}_{50}$  (cytotoxic concentration) values were obtained with GraphPad Prism 5 (GraphPad Software, Inc.) for reference drug and confirmed compounds.

## RESULTS

### High-content screening assay development

The screening was based on the infection of U2OS cells by TCTs. *T. cruzi* trypomastigotes have the ability to infect most, if not all, types of nucleated cells. U2OS are an osteosarcoma cell line considered optimal for high-content image-based screenings due to their growing in monolayers and spread over surfaces, without the need for coating. Also because of its large cytoplasm, it facilitates cell segmentation and accurate parasite detection. Staining with a nucleic acids dye reveals both host cells nuclei and amastigote nuclear and kinetoplast DNA, whose pictures were analyzed with a specifically developed in-house software (Fig. 1). The differences in the size of DNA material was exploited for algorithm development. Infection ratio (IR), defined as the coefficient of number of cells infected by at least one parasite by the number of total cells, was  $0.83 \pm 0.04$  for the 1% DMSO control versus  $0.06 \pm 0.01$  for the benznidazole  $\text{EC}_{100}$  (400  $\mu\text{M}$ ), resulting in a wide IR window that enables accurate analysis and the detection of slight variations in compound activity, an important improvement over other assays where the IR window is of just 30% [23]. These values are clearly visible by the separation of control wells IR values in Fig. 2A and translate into a robust  $Z'$  value of 0.81, conferring a high statistical confidence in the assay. In terms of average number of parasites per infected cell (p/ic), 1% DMSO control had  $14.5 \pm 1.3$  p/ic while  $\text{EC}_{100}$  and non-infected controls displayed  $2.0 \pm 0.1$  and  $2.2 \pm 0.3$  p/ic respectively. These values in negative controls correspond to a limitation of the software that identifies small dotted structures in the pictures as parasites, so for any analysis including the parameter p/ic we took this limitation into account.

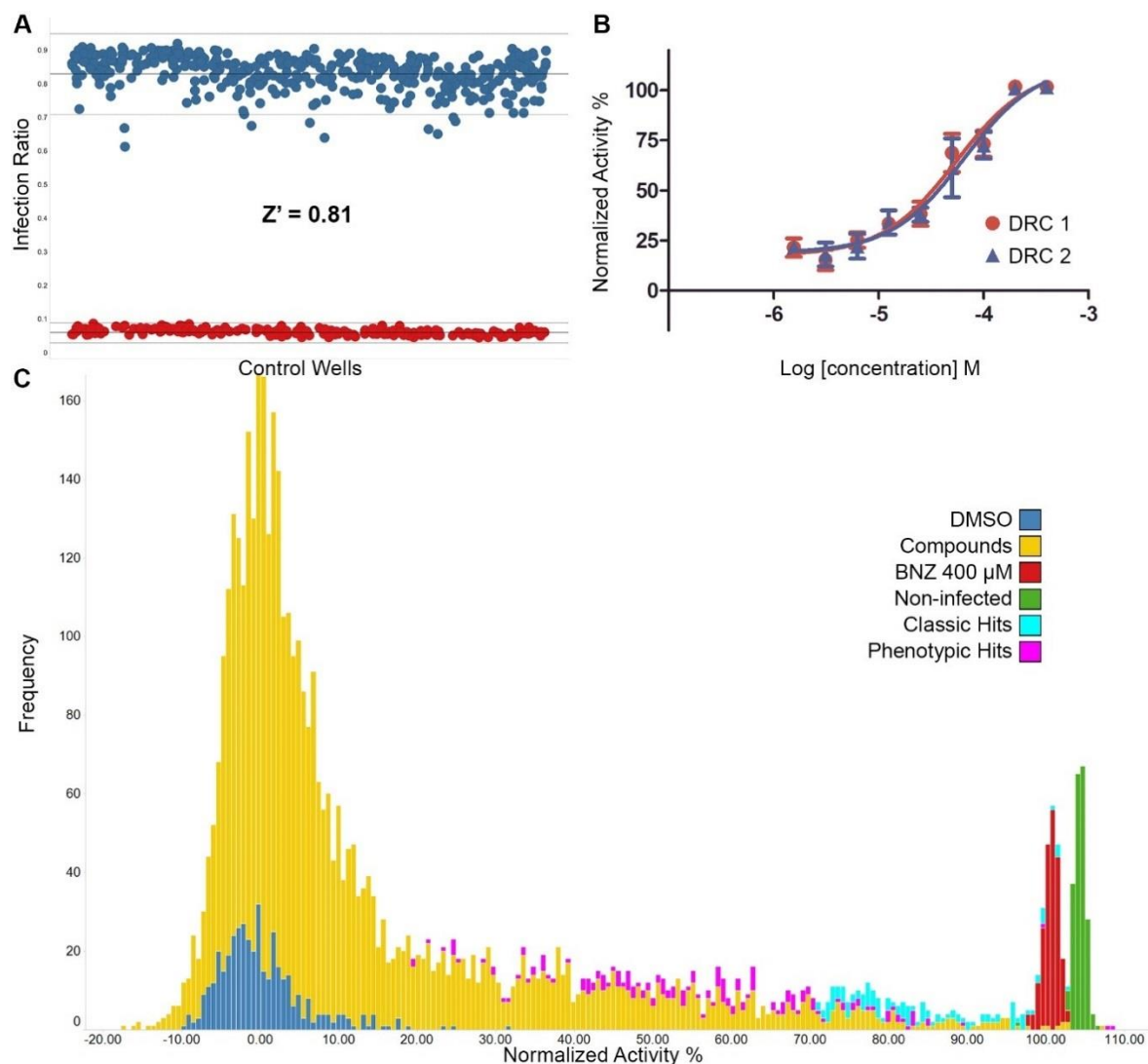


**Figure 1. Software developed in-house for image analysis.** **A)** Interface for plate selection, levels adjustment and picture navigation **B)** Raw images acquired in Operetta automated microscope, showing DNA staining by DraQ5 displayed as white color. **C)** Plugin window developed for *T. cruzi* analysis. **D)** Parameters data obtained from the image analysis: number of total cells, number of infected cells, infected cells/total cells, number of total parasites, average parasites/infected cells, standard deviation of parasites/infected cell. **E)** Image analyzed by the software, showing the detection of nuclei of infected cells (green), cytoplasmic membrane of infected cells (blue), parasites (red) and nuclei of non-infected cells (light blue). **F)** Plate map of the selected screening plate, with a color gradient representing the infection ratio (IR), with blue representing high IR and white representing low IR.

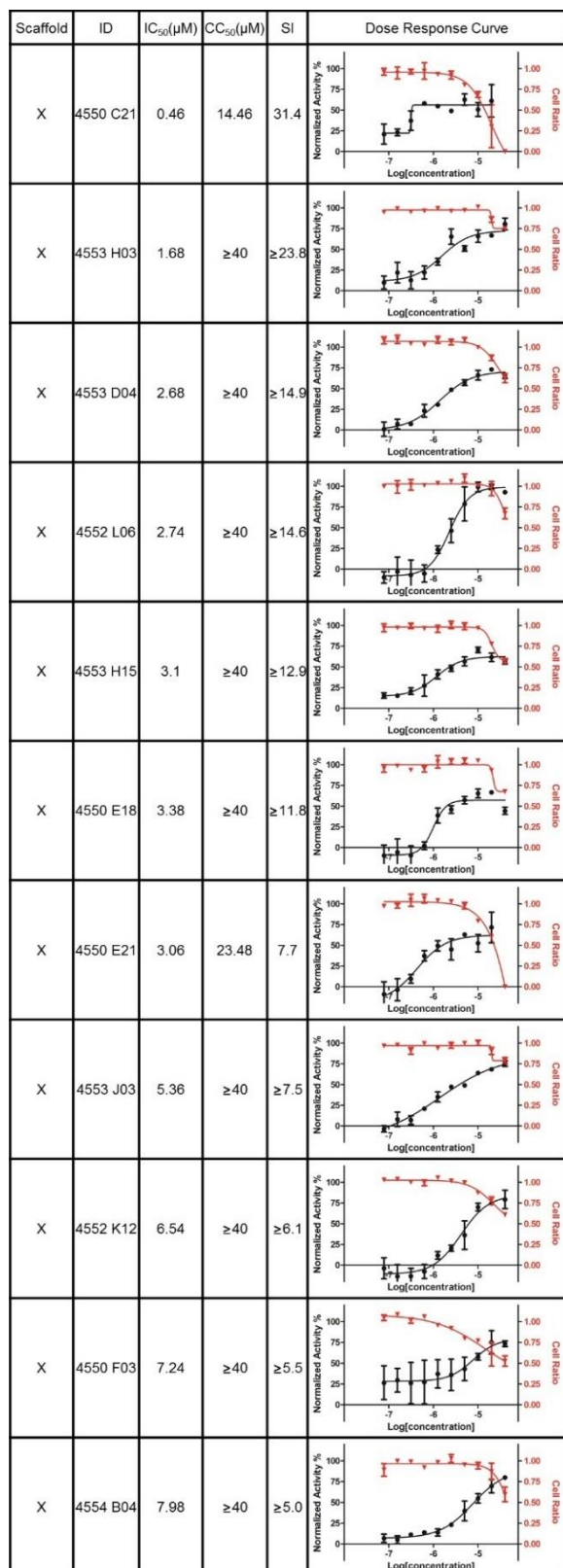
Cell toxicity induced by compounds was quantified by the parameter cell ratio (CR) = compound total cell number / average 1% DMSO total cell number. Cell number is derived by total nuclei count by the software. Average CR for the negatives controls EC100 and non-infected were  $1.53 \pm 0.11$  and  $1.52 \pm 0.13$  respectively. The reported values are higher than 1 because *T. cruzi* infection delays cell division and causes cell death to some extent. The absence of infection either by the presence of an active drug or no addition of parasite contributes to cell preservation. In order to access variability overtime in the screening process, two plates with a dose-response curve (DRC) to benznidazole starting at 400  $\mu\text{M}$  were screened before and after the BioFocus compound plates. The DRCs had  $\text{IC}_{50}$  values of 54.,72 and 65.40  $\mu\text{M}$  (Fig. 2B), values in the range of previous screenings done in-house.

### **Drug library screen, active hits selection and confirmation**

The first selection aimed at identifying hits that were able to clear parasites from infected cells, thus reducing infection ratios. This analysis originated a set of compounds here called classic hits, since traditional drug discovery for intracellular organisms usually uses this parameter as a measure of compound activity. The criteria applied was normalized activity  $\geq 70\%$  and CR  $\geq 0.5$  and originated 119 primary hits. The secondary screening (i.e. DRC) confirmed 76 compounds. Only the compounds that reach at least 70% are considered confirmed hits. To account for assay variability, normalized activity higher than 70% minus 1 standard deviation was considered as a cutoff. The confirmation ratio was calculated to be 64%, in agreement with other screenings carried in-house. Confirmed hits with a selectivity index (SI)  $\geq 2$ , totaling 36 compounds, were clustered in order to access structure-activity relationship. 5 clusters and 2 singlets were grouped in distinct scaffolds (data not shown). The most active hits, selected based on SI  $\geq 5$ , are shown in Table 1 and are characterized by low IC<sub>50</sub> values ( $<10\ \mu\text{M}$ ) and most of them had no CC<sub>50</sub> calculated, since no concentration of the drug reached 50% of cell viability. In those cases the CC<sub>50</sub> was represented by  $>40\ \mu\text{M}$ , and the SI was calculated based on that value.



**Figure 2. High-Content Screening.** **A)** Plot of the infection ratio (Y-axis) for positive (benznidazole at 400  $\mu$ M – red) and negative (1% DMSO – blue) control wells for all screening plates (X-axis);  $Z'$ -factor of 0.81, demonstrating the high statistical confidence of the assay. **B)** Dose-response curves for reference compound benznidazole before (DRC1,  $IC_{50} = 54.72 \mu$ M) and after (DRC2,  $IC_{50} = 65.40 \mu$ M) the screening of the compounds plates. **C)** Histogram of the primary screening, showing the distribution of compounds (Y-axis) by their normalized activity: yellow (library compounds), blue (negative control, 1% DMSO), red (positive control,  $EC_{100}$  benznidazole = 400  $\mu$ M), green (non-infected cells), light blue (classic hits) and magenta (phenotypic hits).



**Figure 3. Dose-response curves of the top hit compounds.** The eleven most active compounds are represented with dose-response curves of normalized activity (black dots and line) and also of cell ratio (red triangles and line). Only confirmed compounds with IC<sub>50</sub>  $\leq$  10  $\mu$ M and selectivity index (S.I.)  $\geq$  5 were chosen. It was not possible to calculate a cytotoxic concentration (CC<sub>50</sub>) for the majority of the compounds (represented by CC<sub>50</sub>  $\geq$  40  $\mu$ M, the highest concentration tested).

### Selection of compounds interfering with parasite intracellular development

A second hit selection was carried in order to identify compounds able to interfere with the parasite intracellular development. The parameter exploited was parasites per infected cell, and as a primary filter, we selected compounds that had only 3 to 6 parasite, on average. This low number of parasites contrasts with the number of about 14 found in the negative control 1% DMSO. After visual confirmation, 192 compounds were selected to be confirmed by 10-point DRC in the same conditions as the primary screening. Instead of plotting normalized activity and cell ratio versus concentration, normalized activity and p/ic versus concentration graphs were plotted. For reference drug and classic hits, as concentration increases the activity increases as well and there is a decrease in the number of p/ic. The profile sought were graphs in which the normalized activity stays relatively constant through concentrations but there is a decrease in the parasite

numbers. In theory, this analysis would enrich for compounds that interfere with intracellular development. The analysis identified 35 such compounds, that were subsequently tested on the high-content immunofluorescence assay developed.

### Immunofluorescence assay development

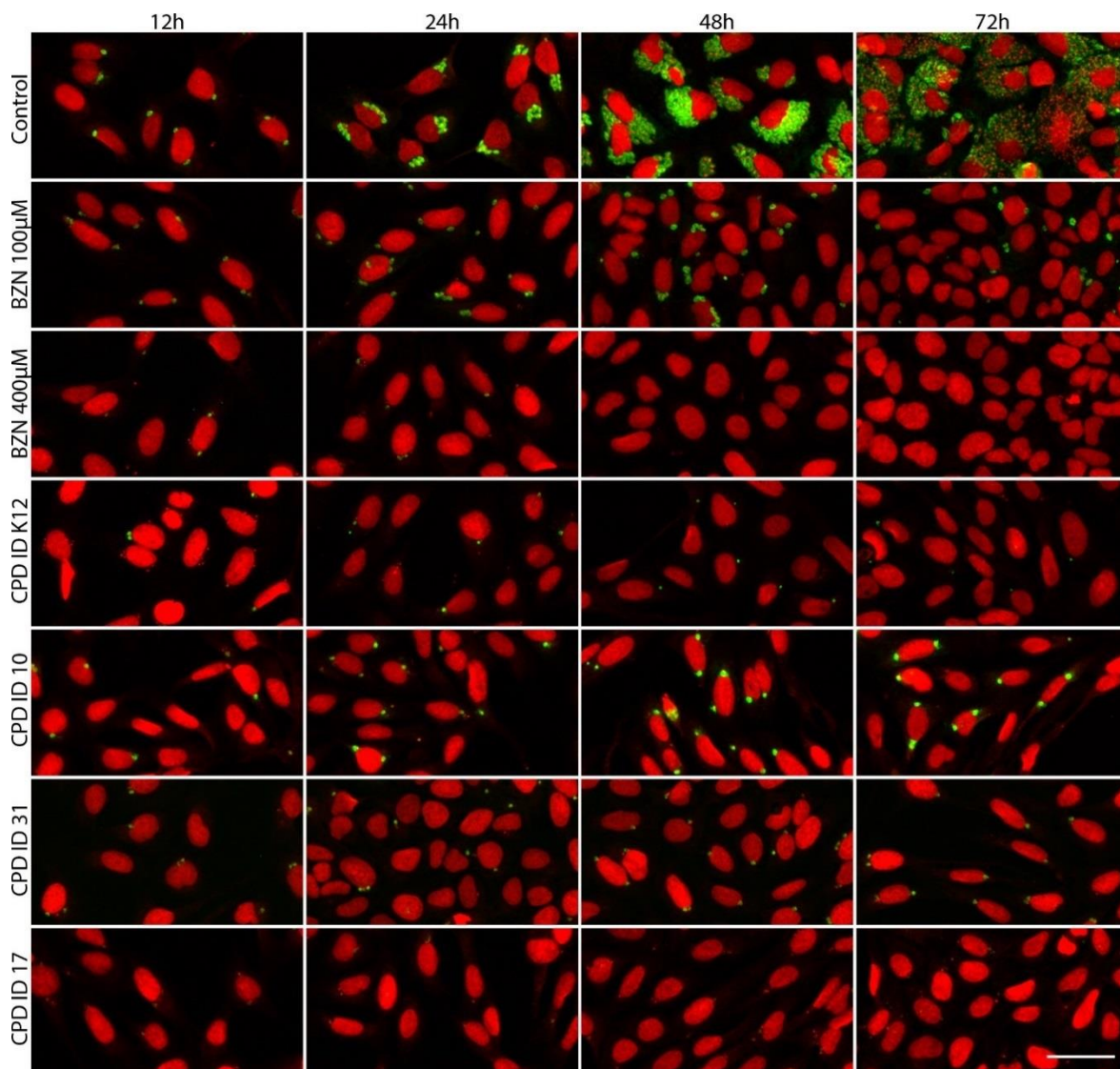
The monoclonal antibodies 2C2 and 39 were both used to study the intracellular cycle of the parasite by selectively binding to *T. cruzi* surface molecules of each parasite stage, amastigote and trypomastigote, respectively. At least 8 concentrations were tested for each compound, and 21 pictures per concentration per time point were visually analyzed. mAb 2C2 starts binding to amastigotes as early as 30 minutes after infection, and mAb 39 scarcely binds to parasites in the first moments of infections, an observation in agreement with previous studies that account for a fast differentiation to trypomastigotes once they enter the cell [24]. Most of the times where was possible to identify parasites positively marked with mAb 39, they had a round shape, indicating the coiling that immediately precedes amastigote differentiation. Several time-points were tested to access representative states of the intracellular cycle of *T. cruzi*, and 12, 24, 48 and 72 hours were chosen (Fig. 4 and 5). 0.5% DMSO was used as a negative control to access the normal development of the parasites. At 12 hours there are present a couple of parasites per cell, most of which are 2C2 marked and have a small round shape. At 24 hours, the amastigotes, now with a stronger 2C2 signal, have gone through 2 or 3 cycles of multiplication. No mAb 39 is identified at this time-point nor at the following, 48 hours. By this time, amastigotes have replicated several times and count about 14-16 per cell. At 72 hours the amastigotes start to differentiate into trypomastigotes once again, and that is visible by the decreased levels of mAb 2C2 expression and the start of expression of mAb 39. As a positive control two concentrations of benznidazole were used, one equal to the EC<sub>100</sub> (400  $\mu$ M) and a sub-lethal concentration (100  $\mu$ M). In the first case, some of the parasites start to express 2C2 in lower levels, but eventually are killed by the compound while many of them don't even show any binding for 2C2, and after the 48 hours time-point, no Draq5 marking for the kinetoplast and parasite nucleus is visible. The 100  $\mu$ M concentration allows the differentiation and hindered replication, but in a much lower rate than control. After 48 hours, there is a decrease in amastigote numbers, indicating a slower killing of the parasite by benznidazole.

Compound K12 is a classic hit tested on the immunofluorescence assay. For this compound, also very few parasites express 2C2, and in much lower levels than the control. After 24 hours the parasites loose viability and only a residual 2C2 marking is visible, most of the times not accompanied by nuclear/kinetoplast Draq5 staining, indicating parasite death.

Of all the 35 compounds tested in the immunofluorescence assay, 22 presented a distinct phenotype when compared to the controls used. We present three of those phenotypes. Compound 10 was characterized by amastigotes that are not able to multiply



and remain in constant numbers up until the last time point. Additionally, they are abnormally strong in 2C2 marking suggesting a continued expression and accumulation of ssp-4 at the parasite surface. This result may be a consequence of the blocking of some parasite differentiation factor not crucial for parasite viability but that enables survivability and persistence in the cell.

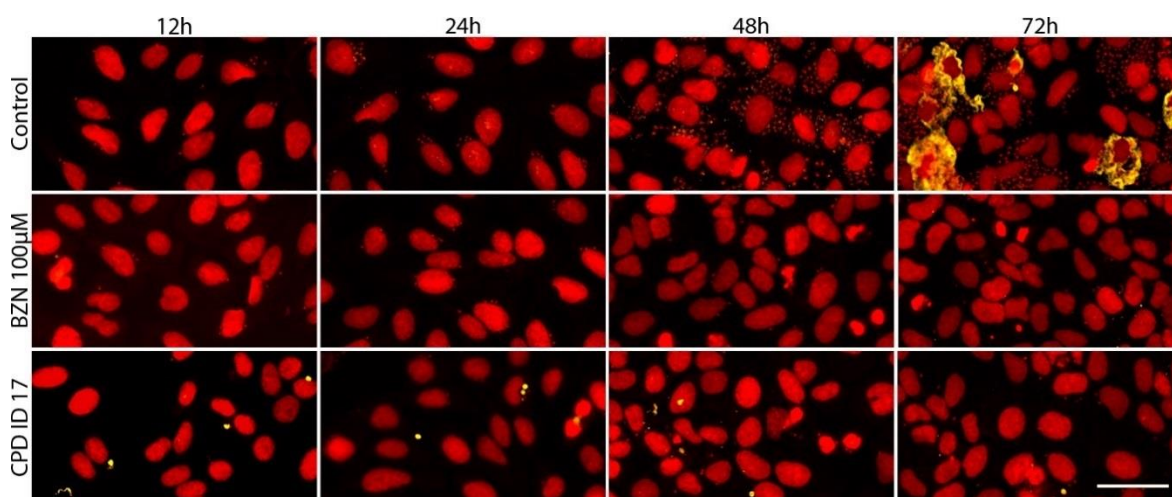


**Figure 4. Phenotypes found with the monoclonal antibody 2C2 (mAb 2C2) immunofluorescence assay.** The images displayed in the panels represent the controls, reference drug and selected compounds for four time-points (12, 24, 48 and 72h) using monoclonal antibody 2C2 against amastigote-specific antigen ssp4.

Compound 31 presents very small amastigotes weakly marked by amastigote antibody throughout all the time-points, very similar in shape and size to the 12 hours 0.5% DMSO control parasites. This may hint at an inhibition of early factor(s) required for amastigote growth and differentiation. Once again, since the parasites are still marked at 72 hours with 2C2, such inhibition is not enough to cause the elimination of the parasite.



Compound 17 was characterized by a total absence of 2C2 in all the time-points, but with indication of parasite presence by Draq5 staining of nuclear and kDNA. The mAb 39 immunofluorescence revealed many DNA spots co-localized with trypomastigote-shaped and round-shaped forms, leading to the hypothesis that there is some sort of trypomastigote stage arrest, not allowing the parasites to differentiate into amastigotes. These trypomastigotes and round-shaped intermediate forms are present up to 48 hours, and are occasionally detected at 72 hours.



**Figure 5. mAb 39 immunofluorescence assay.** The images displayed in the panels represent the controls, reference drug and compound 17 for four time-points (12, 24, 48 and 72h) using monoclonal antibody 39 against trypomastigote-specific antigen TS.

## DISCUSSION

Target-based approaches have been traditionally used not only for parasitic diseases, but also for other diseases drug discovery. Attempts to find a new drug for Chagas disease have followed this trend, with all the current molecules on the pipeline targeting a specific and known parasite protein. One of them is cruzain, also called cruzipain, a target discovered two decades ago and that has been studied extensively ever since [25-28]. K-777, a vinyl sulfone that inhibits this enzyme is currently in prep/clinical studies but other inhibitors are still actively pursued as well [11,29]. Another sought target is 14 $\alpha$ -demethylase, commonly called CYP51, whose similarity with that of pathogenic fungi has led to the repurposing of several anti-fungal azoles with three distinct molecules of this class making it to the pipeline for Chagas disease [10,30,31]. This target still attracts much research, as proven by a recent screening of 104,000 compounds that yielded a potent inhibitor [32].

Although some success has been achieved with target-based approaches, it is considered that cell-based approaches constitute the most promising strategy when it comes to find drugs for parasitic diseases [33]. This is because they resemble the most what happens *in vivo* and ultimately provide the best hits, selected for their ability to selectively kill the parasite. The inhibition of an isolated, purified enzyme under well-defined and controlled conditions, that is the staple of target-based approaches, is far from the complexity of the parasite cellular system. Also, these assays require appropriate target validation by genetic or biochemical means. Because cellular permeability and intracellular metabolic effects are issues not initially addressed, as well as their inability to provide cytotoxic measurements, the attrition rates for target-based approaches are potentially higher. In cell-based assays like the described herein, the concomitant measure of activity and preliminary cytotoxicity evaluation is not only important for screening purposes but also allows for a more focused medicinal chemistry development phase, since only lead compounds with the desired potency and toxicity are prioritized. One potential disadvantage of such assays is the low throughput, but we have successfully screened up to 10,000 compounds per week with minimal automation using the protocol described in this paper. Target deconvolution, although not mandatory, is usually recommended in later development phases for the drugs discovered by this strategy.

Some attempts have been made to develop target-free assays for Chagas disease. One study by Engel et al. used bovine embryo skeletal muscle and Huh-7 cells infected with trypomastigotes and stained with DAPI to screen a known library of 909 FDA approved drugs [23]. Although it represents an advancement face to previous studies, the infection ratios acquired with this protocol did not surpass 30%, and is limited by a 96 well format that offers low throughput. A later study tried to improve host cell segmentation by transfecting mammalian cells with a GFP reporter gene that facilitates boundary identification but that adds a non-natural interference that may potentially affect the assay [34]. Similarly to the previous screening, infection ratios observed were also low and parasite detection was poor.

The screening assay herein described is a considerable advancement over previous protocols because it uses both cells and parasites not genetically modified, uses only one fluorescence measurement and quantifies infection ratio. The statistical Z' value of 0.81 is proof of the robustness of the screening assay.

The screening of the anti-kinase/phosphatase library identified 33 compounds that could be grouped into 7 clusters (data not shown) of active compounds able to clear the infection. All the 33 molecules had a  $IC_{50}$  lower than 20  $\mu M$  with 8 having an  $IC_{50}$  lower than 5  $\mu M$ . The most active compound found had an  $IC_{50}$  of 460 nM. The majority of the top

active compounds also had high  $CC_{50}$ , with the highest concentration tested not reaching a CR of 0.5 and suggesting that selectivity indexes could be even higher. Altogether, none of the compounds presented a selectivity index bigger than 31, with only 11 compounds being bigger than 5.

When we look at the core structures of the majority of the clusters (data not shown), we can clearly see the similarity between their constitutive heterocyclic backbone and the core of a purine, a component of ATP molecules that participate in kinase and phosphatase reactions. ATP-competitive inhibitors are one of the four main groups of kinase inhibitors, and although not the ideal (like activation inhibitors) they comprise the most common class actually in clinical trials [35].

As was previously stated, *T. cruzi* and other trypanosomatids have a comparatively big kinome. One possibility is that while in metazoa and yeast the ultimate targets of many signaling cascades are transcriptions factors which then trigger the expression of new sets of genes, in trypanosomes there is an indiscriminate transcription of most genes in large polycistronic units. Thus signaling cascades mediated by kinases may have a function in post-transcriptional regulation.

Some trypanosomatid kinases have been proved to be essential for parasite viability, but the fact that the compounds may be targeting a host kinase shall not be discarded. For instance, a host cell kinase has been related with *T. cruzi* invasion [36]. It cannot be excluded that the compounds may be targeting host cell kinases responsible for normal cellular functions that the parasite may require. The compounds discovered in this study may be useful not only for *T. cruzi* but also for other trypanosomatids due to kinome identity [13].

Another major advantage of our assay and respective analysis software was the possibility to detect compounds that might be interfering with the parasite intracellular development by making use of all the data provided by the image analysis. Our phenotypic selection yield compounds that displayed distinct phenotypes in relation to stage of the intracellular cycle of *T. cruzi* being affected. The three exemplified compounds displayed specific phenotypes that, as far as we are aware, have not been previously described. Additionally, our results suggest that it may be possible to stop the parasite development in specific stages inside cells. Because *T. cruzi* biology is still poorly understood in comparison to other trypanosomatids, in part due to its complex genetics [37] and also because of absence of functional RNAi interference machinery [38], the finding of such chemical compounds may be useful as biological probes to study the parasite. Questions like what triggers the stage differentiation and the pathways involved, or what factors are associated with parasite intracellular persistence could be answered with the help of such compounds.

Also, the identification of new drug targets by chemical genomics is a possibility. Since many kinases are involved in cell cycle regulation in eukaryotic cells, these could present a theoretical target of our compounds. Other possible future application of such immunofluorescence assay could be the analysis of known and novel compounds active against the parasite, in order to elucidate their mechanism of action. A downside to the assay is the need to test the two antibodies in separate, since they are both monoclonal produced in the mouse. A future improvement can be the development of a high-content immunofluorescence able to simultaneously access amastigote and trypomastigotes stage, either by selecting a different antibody from other source or by differentially labeling of one of the monoclonal antibodies.

## CONCLUSION

This work presented an advanced and optimized HCS assay for Chagas disease that is a step forward compared to previous studies in terms of simplicity, throughput, adaptability and quality of the results. A total of 7 clusters originated from an anti-kinase drug library is reported and should be further studied due to the potential of targeting this parasite kinome. Additionally, our analysis permitted the identification of compounds that were able to interfere with the normal development cycle of the parasite in its intracellular portion, and are then suggested as chemical tools to help elucidate parasite biology.

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#### **4.2.1.2. Enantiomers of nifurtimox do not exhibit stereoselective anti-*Trypanosoma cruzi* activity, toxicity, or pharmacokinetic properties**

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*Published at Antimicrobial Agents and Chemotherapy 2015, volume 59, number 6, pages 3645-3647.*

##### **Objectives of the study:**

Nifurtimox is currently synthesized and administered as a racemic mixture. To test whether one of the enantiomers of nifurtimox was more or less active, toxic or had more advantageous pharmacokinetic properties, a detailed study assessing those characteristics was undertaken.

##### **Main results:**

→ The separated enantiomers, R-nifurtimox and S-nifurtimox, did not show statistically significant different properties in what concerns antiparasitic activity, host cell toxicity, nor displayed distinct pharmacokinetic properties.

##### **Conclusion:**

The study did not support the use of nifurtimox as a separate enantiomer, since any of them had any advantage over the currently used racemic mixture.



## Enantiomers of Nifurtimox Do Not Exhibit Stereoselective Anti-*Trypanosoma cruzi* Activity, Toxicity, or Pharmacokinetic Properties

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With the aim of improving the available drugs for the treatment of Chagas disease, individual enantiomers of nifurtimox were characterized. The results indicate that the enantiomers are equivalent in their *in vitro* activity against a panel of *Trypanosoma cruzi* strains; *in vivo* efficacy in a murine model of Chagas disease; *in vitro* toxicity and absorption, distribution, metabolism, and excretion characteristics; and *in vivo* pharmacokinetic properties. There is unlikely to be any therapeutic benefit of an individual nifurtimox enantiomer over the racemic mixture.

Nifurtimox is one of only two drugs currently registered for the treatment of Chagas disease caused by the parasite *Trypanosoma cruzi*. While nifurtimox is effective when treatment is initiated during the acute stage of infection, its effectiveness in treating the chronic stage of infection is highly variable (1). Treatment with nifurtimox is associated with a high incidence of severe side effects, and as a result, the related nitroheterocycle benznidazole is currently the drug of choice for the treatment of Chagas disease. *In vivo*, different strains of *T. cruzi* have been reported to exhibit differing susceptibilities to nifurtimox, which may contribute to variations in its effectiveness (2, 3).

Nifurtimox, a 5-nitrofuran that undergoes nitroreduction to form the active species, is marketed as a racemate (Lampit); however, information regarding the biological activity and toxicological and pharmacokinetic properties of the individual nifurtimox enantiomers is not available in the scientific literature. With the aim of improving the currently available chemotherapeutic options for treating Chagas disease, we characterized the individual nifurtimox enantiomers for their antitrypanosomal activity against a panel of *T. cruzi* strains and clones *in vitro* and assessed their efficacy in a murine model of acute Chagas disease. We also determined the *in vitro* toxicity profile and *in vitro* and *in vivo* pharmacokinetic properties of each enantiomer relative to those of the racemic mixture.

Nifurtimox racemate was synthesized by Epichem Pty. Ltd., Perth, Australia. Following separation of the enantiomers by supercritical fluid chromatography and recrystallization, their absolute structure was determined by X-ray diffraction (WuXi AppTec Co., Ltd., Shanghai, China). The *R* and *S* enantiomers were assessed against a panel of seven *T. cruzi* strains and clones, each a member of a distinct lineage or discrete typing unit (Table 1). Mammalian and *T. cruzi* cultures and antiparasitic activity assays were conducted as described previously (4). Briefly, *T. cruzi*-infected U2OS monolayers were treated with benznidazole (control), a nifurtimox racemic mixture, or the individual enantiomers for 72 h (DM28c) or 96 h (remaining clones and strains), and the concentration resulting in 50% inhibition of parasite growth (50% effective concentration [EC<sub>50</sub>]) relative to nontreated, infected controls was determined. The EC<sub>50</sub>s of the racemate and each of the enantiomers were in the low micromolar (benznidazole)

to submicromolar (nifurtimox) range, with no significant difference in potency between the enantiomers against the strains and clones tested. A first estimation of cytotoxicity was done by looking at the compound's selectivity toward the parasite; it was measured against the host cells, the human cell line U2OS, by calculating the CC<sub>50</sub>, the ratio of (i) the average number of cells in compound-treated, infected wells to (ii) the average number of cells in nontreated (mock), infected negative controls. We found that differences between the CC<sub>50</sub>s of the enantiomers were not significant when the same strain or clone was considered (Table 1). Cytotoxicity assessment in the noninfected rat skeletal myoblast L6 cell line did not show any difference between the enantiomers either.

*In vivo* efficacy was assessed as previously described (5), by using a murine model of acute Chagas disease with approval from the Animal Ethics Committee of Murdoch University. Female Swiss mice (8 weeks old, *n* = 5 per group) were infected intraperitoneally with 50,000 trypomastigotes (Tulahuen strain), and drug treatment commenced on day 8 postinfection. Nifurtimox racemate or each individual enantiomer was administered by oral gavage (100 mg/kg) once a day for 20 days. At the end of the 20-day dosing period, 5 out of 5 mice in the nifurtimox racemate group had undetectable levels of parasites in their blood as determined

Received 25 December 2014. Returned for modification 23 January 2015.  
Accepted 16 March 2015.

Accepted manuscript posted online 6 April 2015.

Citation Moraes CB, White KL, Brailard S, Perez C, Goo J, Gaspar L, Shackleford DM, Cordeiro-da-Silva A, Thompson RCA, Freitas-Junior L, Charman SA, Chatelain E. 2015. Enantiomers of nifurtimox do not exhibit stereoselective anti-*Trypanosoma cruzi* activity, toxicity, or pharmacokinetic properties. *Antimicrob Agents Chemother* 59:3645–3647. doi:10.1128/AAC.05139-14.

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doi:10.1128/AAC.05139-14

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TABLE 1 EC<sub>50</sub>s of nifurtimox racemate and its purified enantiomers against a *T. cruzi* strain and done panel

Strain or clone	DTU	Nifurtimox racemate			<i>R</i> enantiomer			<i>S</i> enantiomer		
		EC <sub>50</sub> (μM)	CC <sub>50</sub> (μM) <sup>a</sup>	SI <sup>b</sup>	EC <sub>50</sub> (μM)	CC <sub>50</sub> (μM)	SI	EC <sub>50</sub> (μM)	CC <sub>50</sub> (μM)	SI
Dm28c	I	3.41	111.6	32.7	4.00	104.0	26.0	3.32	101.0	30.4
Y	II	1.00	161.6	161.6	0.65	167.4	257.5	0.69	143.2	207.5
ARMA13 cl1	III	0.91	68.5	75.3	0.78	54.8	70.3	0.71	67.2	94.6
ERA cl2	IV	0.58	124.4	214.4	0.75	136.8	182.4	0.42	108.3	257.9
92.80 cl2	V	0.33	79.5	240.9	0.35	79.7	227.7	0.28	75.7	270.4
Tulahuen	VI	0.32	117.7	367.8	0.32	120.0	375.8	0.25	107.1	428.4
CL Brenner	VI	0.76	63.8	83.9	0.65	58.3	89.7	0.92	74.9	81.4

<sup>a</sup> CC<sub>50</sub> was measured against the U2OS cell line as described in Materials and Methods.<sup>b</sup> SI, selectivity index, defined as the CC<sub>50</sub>/EC<sub>50</sub> ratio.

microscopically, compared to 4 out of 5 and 5 out of 5 in the *R* and *S* enantiomer groups, respectively. To assess potential cures, the parasitemia-negative animals in each group underwent up to three rounds of immunosuppression with cyclophosphamide (50 mg/kg administered intraperitoneally once daily for 4 days, followed by 3 days of rest); at the end of the immunosuppression phase, a rebound of parasitemia was observed in all of the animals, indicating that there were no complete cures when using this model.

*In vitro* toxicity tests included inhibition of the human ether-à-go-go-related gene (hERG) potassium channel and mutagenic potential. For the hERG inhibition assay, an automated patch clamp method (QPatch<sup>HTX</sup>; WuXi AppTec Co., Ltd., Shanghai, China) was used and indicated that neither of the enantiomers exhibited detectable inhibition of the channel (50% inhibitory concentration [IC<sub>50</sub>] > 30 μM). A mini-Ames assay (WuXi AppTec Ltd., Suzhou, China) using *Salmonella enterica* serovar Typhimurium strains TA98 and TA100 in the presence or absence of exogenous metabolic activation (Aroclor 1254-induced rat liver S9 mix) turned out positive for each enantiomer (>2-fold increase in the mean number of revertant colonies); a similar increase in the number of revertants per plate (reverse mutations at the selected loci of the two tester strains) in the absence or presence of S9 activation was exhibited by each enantiomer.

Physicochemical and metabolic stability properties and CYP inhibition were assessed as described previously (5). Both enantiomers exhibited good aqueous solubility (50 to 100 μg/ml) and moderate lipophilicity (a distribution constant [log D<sub>pH 7.4</sub>] of 2.1) and were metabolically stable in the presence of human hepatic microsomes (*in vitro* intrinsic clearance of <7 μl/min/mg of protein). The enantiomers both showed minimal inhibition of cytochrome P450 3A4, with an IC<sub>50</sub> of >20 μM (data not shown).

The *in vivo* pharmacokinetic properties of (*R*)- and (*S*)-nifurtimox were investigated in male Sprague-Dawley rats using protocols approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee and in accordance with procedures outlined in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Each enantiomer was administered intravenously (i.v.) by constant-rate infusion (3 mg/kg) over 10 min into a catheter surgically inserted into the jugular vein under isoflurane anesthesia. Oral doses (10 mg/kg) were administered as an aqueous suspension by oral gavage. Blood samples were collected for up to 24 h postdose and analyzed by liquid chromatography-mass spectrometry. Following i.v. administration, plasma (*R*)- and (*S*)-nifurtimox concentrations rapidly declined to levels below the analytical lower limit of quantitation;

however, within the first 6 h, there was no discernible difference in i.v. exposure between the two enantiomers. Similarly, after oral administration, there was no substantial difference between the profiles of (*R*)- and (*S*)-nifurtimox during the initial 5-h period, and while concentrations of the *R* enantiomer at later sample times were greater than those of (*S*)-nifurtimox, there was no major difference in the overall exposure of the two enantiomers (Fig. 1).

In summary, these studies reveal no evidence of nifurtimox enantiomer stereoselectivity in relation to *in vitro* anti-*T. cruzi* activity against multiple strains and clones or *in vivo* in a murine model of acute Chagas disease. For selected toxicity parameters,

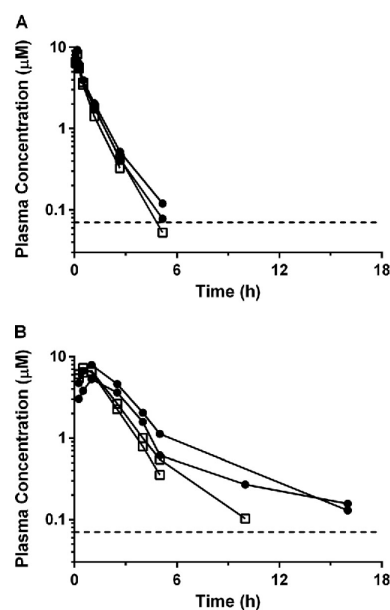


FIG 1 Plasma concentration-versus-time profiles of (*R*)- and (*S*)-nifurtimox in male Sprague-Dawley rats following i.v. (3 mg/kg, panel A) or oral (10 mg/kg, panel B) administration. Data are shown for two individual animals per compound per dose and route. (*R*)-Nifurtimox is represented by filled symbols, and (*S*)-nifurtimox is represented by open symbols. The dashed line in each panel represents the analytical lower limit of quantitation.

both enantiomers exhibited no detectable inhibition of the hERG potassium channel, and both were similarly positive in a mini-Ames mutagenicity test. There was no detectable difference between the physicochemical, metabolic, or pharmacokinetic properties of the two enantiomers. The results suggest that there is unlikely to be any therapeutic benefit in relation to safety or efficacy of an individual nifurtimox enantiomer over the racemic mixture.

#### ACKNOWLEDGMENTS

This work was supported by DNDi and the National Research Foundation of Korea (NRF) grant funded by the Korean Government (MSIP, no. 2007-00559), Gyeonggi-do, and KIST. For the work described in this paper, DNDi received financial support from the following donors: the Directorate General for International Cooperation (DGIS), The Netherlands; the Department for International Development (DFID), United Kingdom; Médecins Sans Frontières (Doctors without Borders, MSF), International; the Reconstruction Credit Institution-Federal Ministry of Education and Research (KfW-BMBF), Germany; and the Swiss Agency for Development and Cooperation (SDC), Switzerland. The donors had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. L.G. was supported by the Fundação para a Ciência e a Tecnologia through grant SFRH/BD/81604/2011.

Racemic nifurtimox and the separate *R* and *S* enantiomers were sup-

plied by Epichem Pty. Ltd., Perth, Australia, and their contribution is gratefully acknowledged.

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#### **4.2.1.3. Synthesis of new 7-aryltioetherthieno[3,2-b]pyridines bearing arylamides or 1,2,3-triazoles 1,4-disubstituted and evaluation of their antiparasitic activity against *Leishmania infantum*, *Trypanosoma brucei* and *Trypanosoma cruzi***

Joana F. Campos, Gabriela Cristo, Luís Gaspar, Agathe Begouin, Catarina P. Baptista, Maria-João R. P. Queiroz and Anabela Cordeiro-da-Silva

*Manuscript in preparation*

##### **Objectives of the study:**

The present study focused on the synthesis of new 7-arylthioetherthieno[3,2-b]pyridine derivatives and their evaluation as antiparasitic agents against *L. infantum*, *T. brucei* and *T. cruzi*, with the last parasite being assayed with an HCS protocol.

##### **Main results:**

→ Seven new chemical entities belonging to two different scaffolds were synthesized and evaluated against three trypanosomatids. The antiparasitic activity of the derivatives was superior for both *Trypanosoma* species, whereas no significant activity was found against *L. infantum* parasites.

##### **Conclusion:**

In conclusion, although the newly synthesized compounds display some antiparasitic activity, new derivatives with increased potency and higher selectivity should be pursued.

## **Synthesis of new 7-aryltioetherthieno[3,2-*b*]pyridines bearing arylamides or 1,2,3-triazoles 1,4-disubstituted and evaluation of their anti-parasitic activity against *Leishmania infantum*, *Trypanosoma brucei* and *Trypanosoma cruzi***

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## Abstract

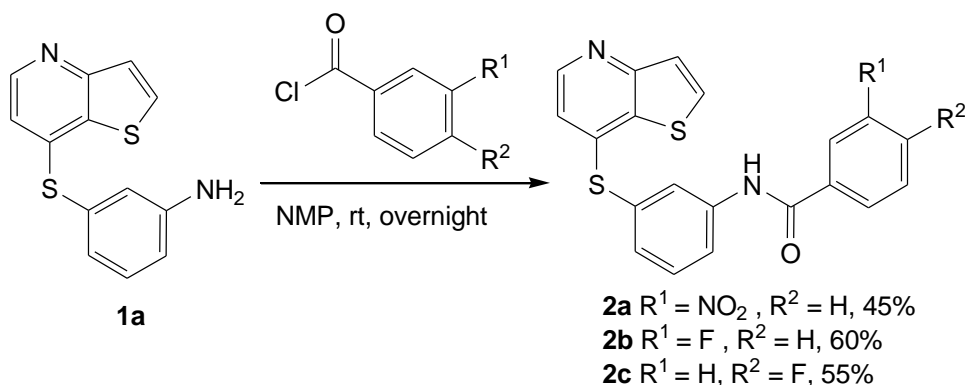
New 7-arylthioetherthieno[3,2-*b*]pyridines bearing arylamides or 1,2,3-triazoles were synthesized by reaction of 3-(thieno[3,2-*b*]pyridin-7-ylthio)aniline either with benzoyl chlorides to give the first or, in one pot procedure *via* the corresponding azide by a Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) to give the latter. The anti-parasitic activity of the compounds obtained was evaluated at 10  $\mu$ M against *Leishmania infantum*, *Trypanosoma brucei* and *Trypanosoma cruzi*. The diarylamide with a NO<sub>2</sub> group in the *meta* position relatively to the carbonyl of the amide presented anti-parasitic activity against both *T. brucei* and *T. cruzi* and a F atom in the *para* position resulted in high activity for *T. brucei* and *L. infantum* but only moderate for *T. cruzi*. When the amide linkage was replaced by a 1,2,3-triazole 1,4-disubstituted moiety no activity for *L. infantum* was observed for the tested compounds; for the compound with a phenyl group in the triazole high activity was shown for *T. cruzi* and only moderate was observed for *T. brucei*; the compound with a F atom in the *meta* position showed activity for both *T. brucei* and *T. cruzi*; the substitution with a pyridine or a thiophene, resulted in a high activity for both *T. brucei* and *T. cruzi* only for the thiophene derivative. Nevertheless, at the tested concentration the compounds did not show toxicity using MTT assay in PMA-differentiated THP-1 cells. However NOAELs indicate that selectivity may be improved.

**Keywords:** 7-arylthioetherthieno[3,2-*b*]pyridines, 1,2,3-triazoles, diarylamides, anti-parasitic activity, cytotoxicity.

Parasitic diseases caused by trypanosomatids such as *Leishmania* spp., *Trypanosoma cruzi* and *Trypanosoma brucei* are a group of communicable diseases mostly affecting people in developing countries. These diseases are responsible for a major part of the global morbidity, mortality and poverty.<sup>1</sup> There is still no vaccine available and chemotherapies are far from satisfactory due to the emergence of resistance, toxicity-related side effects, and lack of efficacy. As a consequence, the development of new drugs to treat these diseases is an urgent need.<sup>2,3</sup>

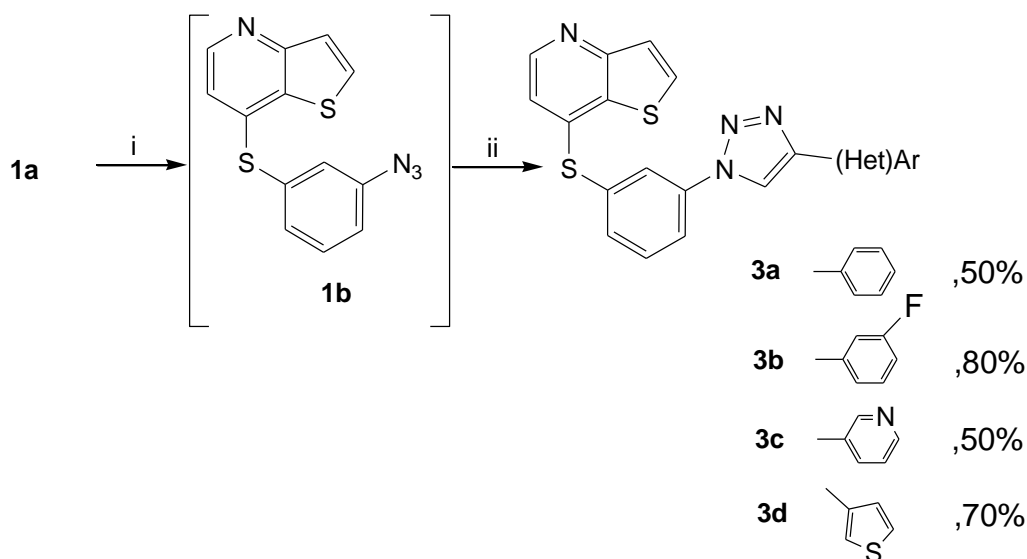
The thieno[3,2-*b*]pyridine derivatives have shown several biological activities.<sup>4</sup> The amide and 1,2,3-triazoles 1,4-disubstituted linkages are also present in different bioactive compounds.<sup>5</sup> Our research group has been interested in the synthesis of thieno[3,2-*b*]pyridine derivatives, namely as antitumor<sup>6</sup> and antiangiogenic compounds.<sup>7</sup> Our previous results had led us to search for new molecules with trypanocidal activity. Here we present the synthesis of new di(hetero)arylthioethers in the thieno[3,2-*b*]pyridine series, all substituted in the meta position relative to the thioether by arylamides or 1,2,3-triazole 1,4-disubstituted moieties. The anti-parasitic activity of the compounds was evaluated against *Leishmania infantum*, *Trypanosoma brucei* and *Trypanosoma cruzi*.

From the 3-(thieno[3,2-*b*]pyridin-7-ylthio)aniline (**1a**), previously prepared by us,<sup>7</sup> arylamides **2a-2c** and 1,2,3-triazoles 1,4-disubstituted **3a-3d** were synthesized either by reaction with benzoyl chlorides in *N*-methylpyrrolidinone (NMP) at RT overnight<sup>8</sup> to give the first in moderate to good yields (Scheme 1) or, in a two steps one pot procedure using the mild conditions described in 2007 by Moses et al.,<sup>9</sup> with *tert*-butyl nitrite (*t*-BuONO) and azidotrimethylsilane (TMSN<sub>3</sub>) to form the corresponding azide intermediate, followed by the addition of CuSO<sub>4</sub>·5H<sub>2</sub>O, sodium ascorbate and (hetero)arylalkynes by a Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC),<sup>10</sup> to give the latter in good to high yields (Scheme 2). The azide **1b** was isolated once in excellent yield (92%) and was fully characterized.



**Scheme 1** Synthesis of di(hetero)arylthioethers **2a-2c**.





i: *t*-BuONO (1.5 equiv.), TMSN<sub>3</sub> (1.2 equiv.), CH<sub>3</sub>CN, 0 °C to rt, 5-6h.

ii: (Hetero)aryllalkyne (1.0 equiv.), CuSO<sub>4</sub> (10 mol%), sodium ascorbate (2.0 equiv.), CH<sub>3</sub>CN, rt.

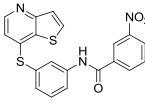
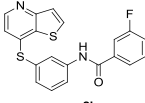
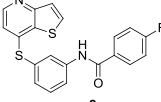
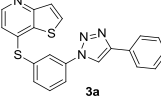
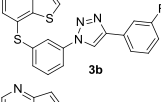
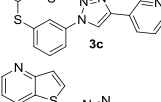
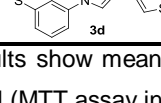
**Scheme 2** Synthesis of 1,4-disubstituted (hetero)aryl-1,2,3-triazole thieno[3,2-*b*]pyridine-7-aryltioethers **3a-3d**.

The Table 1 summarizes the results obtained for the anti-parasitic activity of compounds **2a-2c** and **3a-3d** at 10 µM concentration. From the results we can conclude that the diarylamide **2a** with a nitro group in the *meta* position relatively to the carbonyl group presents a strong inhibitory activity for *T. brucei* (102±0% inhibition) and *T. cruzi* (95±3% inhibition) and no activity for *L. infantum*. When a F atom is placed in the same position of the nitro group in amide **2b**, there was a decrease of the inhibitory activity for *T. brucei* (26±9% inhibition) and *T. cruzi* (52±10% inhibition). Similarly, **2b** showed no activity against *L. infantum*. The change of the F atom to the *para* position in amide **2c**, gave a high inhibitory activity for *T. brucei* (102±0% inhibition), an almost complete inhibition for *L. infantum* (81±5% inhibition), and a moderate activity for *T. cruzi* (52±10% inhibition). With respect to toxicity, these amides showed some cytotoxicity with NOAEL (no observed adverse effect level) > 12.5 µM for **2a**, **2c** and > 25 µM for **2b**.

When the amide linkage is replaced by a 1,2,3-triazole 1,4-disubstituted moiety, no anti-leishmanial activity was found for all the compounds. For compound **3a** with a phenyl group at the 4-position of the triazole, a high inhibitory activity was found for *T. cruzi* (97±1% inhibition) and a moderate inhibitory activity was observed for *T. brucei* (64±0% inhibition). Compound **3b** with a F atom in the *meta* position showed high inhibitory activity for *T. brucei* and for *T. cruzi* (98±0% and 81±4% inhibition, respectively). The substitution with a pyridine or a thiophene in their 3-positions, **3c** and **3d**, respectively, resulted in a high inhibitory activity for *T. brucei* and for *T. cruzi* (97±1% and 80±3% inhibition, respectively) only for compound **3d**. Compound **3c** showed a moderate activity against *T. brucei* (61±15%

inhibition) and a mild activity against *T. cruzi* (31±6% inhibition). With respect to toxicity, these triazoles show a clear tendency, with compound **3b** with a F atom in the *meta* position showing high cytotoxicity with NOAEL > 12.5 µM, followed for compound **3d** showing NOAEL > 25 µM, and compounds **3a** and **3c** that are less toxic with NOAEL > 50 µM and 100 µM, respectively.

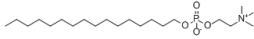
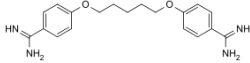
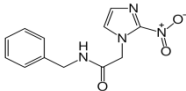
**Table 1.** Anti-parasitic activity against *Leishmania infantum* intracellular amastigotes, *Trypanosoma brucei* bloodstream forms, *Trypanosoma cruzi* amastigotes and cytotoxicity against THP1-derived macrophages (NOAEL).

Compound	10 µM single dose testing (%activity ± SD)			Toxicity NOAEL (µM)
	<i>Leishmania infantum</i> intracellular amastigotes	<i>Trypanosoma brucei</i>	<i>Trypanosoma cruzi</i>	
 2a	N A	102 ± 0	95 ± 3	> 12.5
 2b	N .A	26 ± 9	52 ± 10	> 25
 2c	81 ± 5	102 ± 0	47 ± 1	> 12.5
 3a	N.A.	64 ± 0	97 ± 1	> 50
 3b	N.A.	98 ± 0	81 ± 4	> 12.5
 3c	N.A.	61 ± 15	31 ± 6	> 100
 3d	N.A.	97 ± 1	80 ± 3	> 25

Results show means activities of at least three independent assays. NOAEL= No-Observed Adverse Effect Level (MTT assay in PMA-differentiated THP-1 cells). N.A., No Activity.

All the compounds that presented more than 50% of activity towards any of the parasites were additionally tested at 1 µM. None of the compounds tested at this lower concentration were active against any of the parasites (data not shown). These results suggest that the inhibitory activity is a consequence of nonspecific toxicity mechanisms. All the assays were controlled with a reference drug for each of the diseases as seen in Table 2, miltefosine for *Leishmania infantum*, pentamidine for *Trypanosoma brucei* and benznidazole for *Trypanosoma cruzi*.

**Table 2.** *In vitro* activity of reference drugs for *Leishmania infantum*, *Trypanosoma brucei*, *Trypanosoma cruzi* and cytotoxicity against mammalian cells

Compound	IC <sub>50</sub>			Cytotoxicity
	<i>Leishmania infantum</i> intracellular amastigotes	<i>Trypanosoma brucei</i>	<i>Trypanosoma cruzi</i>	
 Miltefosine	2.9 ± 0.5 µM	N. A.	N. A.	15.9 ± 1.2 µM*
 Pentamidine	N. A.	2.94 ± 0.74 nM	N. A.	47.8 ± 3.3 µM*
 Benznidazole	N. A.	N. A.	1.23 ± 0.30 µM	> 100 µM†

\* THP-1 -derived macrophages; †C2C12 cardiomyocytes

## Conclusions

In this paper we describe the synthesis of new 7-aryltioetherthieno[3,2-*b*]pyridines bearing arylamides or 1,2,3-triazoles 1,4-disubstituted moieties and their biological activity against important disease causing parasites, the trypanosomatids *Leishmania infantum*, *Trypanosoma cruzi* and *Trypanosoma brucei*. The compounds synthesized were generally active against the parasites at both extracellular and intracellular stages. However, some of the compounds had also a NOAEL close to the dose tested in the parasitic assays (10 µM), hinting at nonspecific toxicity mechanisms and low selectivity towards trypanosomes. More derivatives should be synthesized in order to increase potency, selectivity or both.

## Acknowledgments

To the Foundation for the Science and Technology (FCT-Portugal) for financial support through the NMR Portuguese network (Bruker 400 Avance III-Univ Minho). FCT through the grants attributed to A.B. (SFRH/BPD/36753/2007) and L.G. (SFRH/BD/81604/2011), both also financed by POPH (Programa Operacional do Potencial Humano) and FSE (Social European Fund). The research leading to these results has received funding from the European Community's Seventh Framework Programme under grant agreements No.602773 (Project KINDRED).

**Supporting Information** contains the experimental part for chemistry synthesis, including the reaction conditions and characterization of the compounds prepared as well as the description of the materials and methods used in biologic activity assays.

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#### 4.2.1.4. Exploiting the antiparasitic activity of naphthalimides derivatives

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*Accepted for publication at International Journal of Chemistry and Pharmaceutical Sciences (2015)*

##### **Objectives of the study:**

This work aimed at the characterization of the antiparasitic activity of a series of single naphthalimides against *L. infantum*, *T. cruzi* and *T. brucei*. The determination of the structure activity for the various derivatives was pursued.

##### **Main results:**

→ Most derivatives have low or no activity against both intracellular parasites *L. infantum* and *T. cruzi*.

→ Overall, the derivatives were mostly active against bloodstream forms of *T. brucei* parasites, in particular the compounds containing primary alkylamines. Structure activity relationship was only observed for urea derivatives.

##### **Conclusion:**

The presence of just one naphthalimide group seems to drastically decrease the antiparasitic activity of the molecules, a fact particularly noted against *T. brucei* parasites. Among the single naphthalimides, the group of derivatives synthesized with primary alkylamines and un-substituted ureas seems to be the most promising against *T. brucei*.

## Exploiting the Antiparasitic Activity of Naphthalimides Derivatives

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### ABSTRACT

A set of 1,8-naphthalimides derivatives were synthesized and tested against three protozoans that cause important human diseases: *Leishmania infantum*, *Trypanosoma brucei* and *Trypanosoma cruzi*. Additionally, toxicity was determined by growth inhibition of THP-1 derived macrophages. The results suggest that chemical modifications in the carbon chain linking the naphthalimide and the substituting groups have different effects in the parasites. This work should provide new insights for the design and optimization of more potent and directed naphthalimide derivatives against these organisms.

**Keywords:** Naphthalimides derivatives, antiparasitic activity, cytotoxicity.

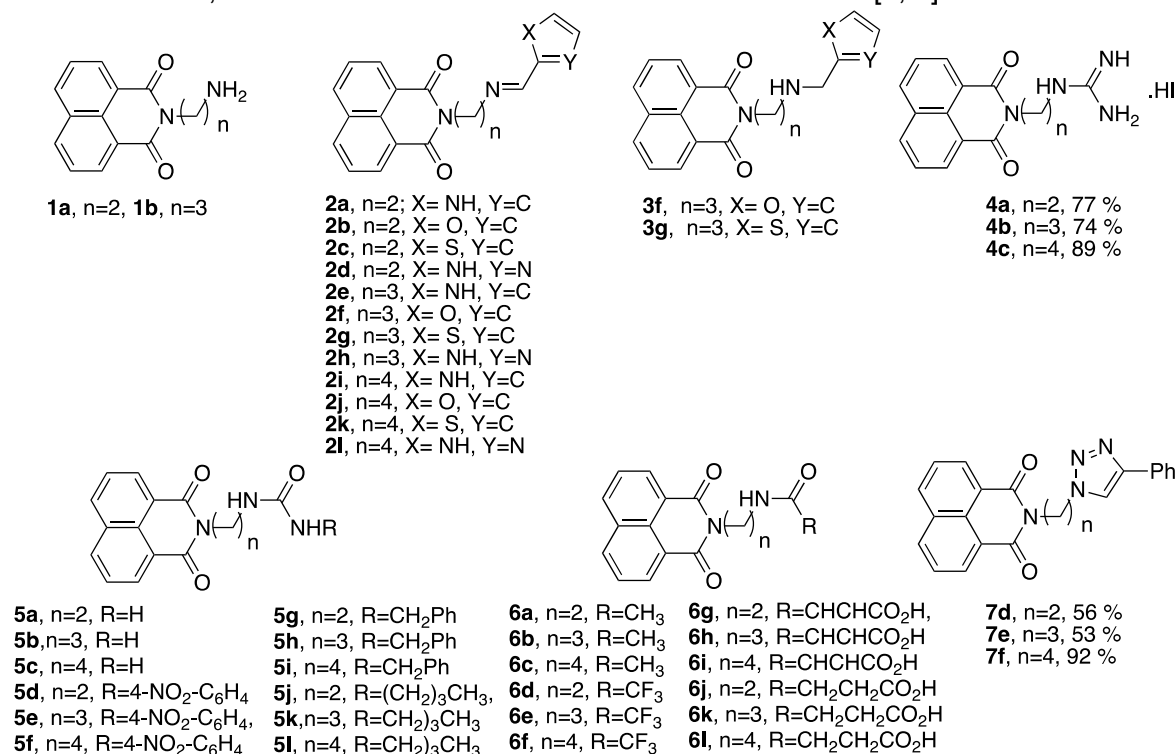
### MAIN TEXT

Parasitic diseases caused by trypanosomatids are still an important health problem, mainly in tropical and subtropical areas. In addition, temperate regions of our globe, including North America and the Asia-Pacific region are also affected by disease-causing protozoans like *Leishmania spp.*, *Trypanosoma cruzi* and *Trypanosoma brucei*. These are the species of trypanosomatids most associated with human health, being responsible for high mortality and morbidity [1]. There are still no vaccines and the actual chemotherapies are far from satisfactory, owing to the emergence of resistances, serious side effects, and its limited efficacy. Therefore, it is imperative to continue the discovery of new drugs to treat these diseases [2].

Naphthalimides and bis-naphthalimides are classes of compounds bearing aromatic groups that have generated intense interest by scientists around the world because of their many reported biological activities. Special attention has been devoted to the high anticancer activity of naphthalimides, which is due to their interactions with DNA by a mechanism of intercalation [3-5]. In addition, recent studies showed that 1,8-naphthalimide derivatives also demonstrated other biological activities, such as antitrypanosomal [6]. In this context, this work proposes to study the relationship between the structure and the activity of a set of 1,8-naphthalimide derivatives with 2, 3 or 4 carbons linking the naphthalimide moiety to different functional groups (amine, imine, guanidine, urea, amide, and 1,2,3-triazole) against three trypanosomatids: *Leishmania infantum*, *Trypanosoma brucei* and *Trypanosoma cruzi*. These 1,8-naphthalimide derivatives compounds had been recently synthesized and include naphthalimidoalkylamines **1a,b**, and its heterocyclic imine



derivatives **2a-l**, heterocyclic amine derivatives **3f,g**, guanidine derivatives **4a-c**, urea derivatives **5a-l**, amide derivatives **6a-l** and triazol derivatives **7d-f** [7, 8].



**Figure 1** - Naphthalimides compounds **1a,b**, **2a-l**, **3f,g**, **4a-c**, **5a-l**, **6a-l**, **7d-f** used in trypanosomatids tests: *Leishmania infantum*, *Trypanosoma brucei*, and *Trypanosoma cruzi*.

All the naphthalimides compounds stock solutions were prepared in DMSO in a concentration of 10 mM and stored at -20 °C. Anti *T. cruzi* (Y strain) activity was performed by high content screening (adapted from [9]); for *T. brucei* (L427 Wild Type) bloodstream forms and axenic amastigote of *L. infantum* (clone MHOM/MA671TMA-P263) activity was determined using the resazurin-based assay [10, 11]; intracellular amastigote activity of *L. infantum* was performed by luciferase assay (adapted from [12]). The cellular toxicity of the compounds was evaluated on THP-1 differentiated macrophages using the MTT assay [13]. The table summarizes the results obtained for the compounds with relevant antiparasitic activity at 10 µM concentration. Overall, our results show that the best activity was against *T. brucei*. The group of naphthalimidoalkylamines represented by compound **1a** and **1b** show strong inhibition at concentration 10 µM (101±1% inhibition); however compound **1a** presents higher toxicity with NOAEL (no observed adverse effect level) > 25 µM in comparison with **1b** NOAEL > 100 µM, suggesting that shorter a carbon chain (n = 2) increases the cytotoxicity. The group of heterocyclic imines compounds **2a-l** generally reveals good activity against *T. brucei*. Within the imino furan subgroup, compounds **2b**, **2f** and **2j**, only **2f** with 3 carbon atoms chain shows to be active (101 ± 1% inhibition), and in the subgroup incorporating imidazole (**2d**, **2h** and **2l**) compound **2l**, with 4 carbon atoms chain, is the least active. The toxicity for heterocyclic imines is lower with 3 and 4 carbon chains. The compound with the best balance activity-toxicity belongs to compound **2e**, with a three carbon atom chain and a pyrrole unit. Heterocyclic amines compounds are also active against *T. brucei*, our results showing almost complete inhibition with compound **3f** (99 ± 1% inhibition) and **3g** (96 ± 3% inhibition). However, compound **3g** shows lower cytotoxicity than **3f**, with NOAEL > 50 µM and > 25 µM, respectively. Guanidine substituted compounds **4a-c** also reveal some anti-*T. brucei* activity, with a clear tendency for toxicity to decrease with the increase of the carbon chain length. Within the urea-substituted compounds **5a-l** the most active compounds against *T. brucei*, at the concentrations tested, is **5f** and **5l** both with 4 carbon atom chains. Within this group, when the carbon chain

increases up to four carbon atoms, so does the activity increase. Also the nature of the terminal groups in compounds **5a-l** is also found to be important for activity. Comparing the cytotoxicity of the four carbon chain members in this group (**5c**, **5f**, **5i** and **5l**), only compound **5c** was found non toxic, suggesting that unsubstituted ureas are less toxic than substituted ureas against THP-1 cell line. In relation to the triazole group, only compound **7f** shows activity against *T. brucei* ( $90 \pm 3\%$  inhibition), with toxicity increasing when the triazole group is linked by a 3 carbon atoms chain. Regarding *T. cruzi*, amine **1a** and guanidine **4a** shows modest antiparasitic activity ( $34 \pm 9\%$  inhibition and  $45 \pm 9\%$  inhibition, respectively). Urea **5l** bearing a 4 carbon atoms chain and a *n*-butyl group as substituent shows to be the most active compound of all tested against *T. cruzi*, ( $81 \pm 8\%$  inhibition). However, all compounds that show some activity against *T. cruzi* are accompanied by an increase in cytotoxicity, suggesting a nonspecific mechanism. Previous studies conducted in our laboratory have shown that bisnaphthalimidopropyl (BNIP) derivatives compounds exert significant effects against *L. infantum* [14]. Surprisingly, no significant anti-leishmanial activity was found for all the groups studied; either against *L. infantum* intracellular and axenic amastigotes. A hypothesis is that the activity is dependent on a second naphthalimide group in the molecule. Overall, the toxicity of the synthesized compounds with a 2 carbon atoms linker presents the higher toxicity. An exception was observed with the substituted urea-derived group, where the 4 carbon atoms chain compounds are the most toxic, with the exception urea **5c**. The group of amides **6a-l** compounds does not present relevant antiparasitic activity (data not shown).

In conclusion, naphthalimides bearing primary alkylamines and un-substituted ureas are the hits for further research in *Trypanosoma brucei*, in combination with longer linkers, and O-, N-functionalized linkers. No interesting results have been found to the other two trypanosomatids studied.

### Table

Antiparasitic activity against *Leishmania infantum* intracellular amastigotes and axenic amastigotes, *Trypanosoma brucei*, *Trypanosoma cruzi* and cytotoxicity in THP1-derived macrophages for the compounds tested.

Functional groups: naphthalimidoalkylamines		10µM single dose testing (% activity ± SD)				Toxicity NOAEL (µM)
Compound	Radical / Carbon number	<i>Leishmania infantum</i> intracellular amastigotes	<i>Leishmania infantum</i> axenic amastigotes	<i>Trypanosoma brucei</i>	<i>Trypanosoma cruzi</i>	
<b>1a</b>	R=NH <sub>2</sub> / n=2	23 ± 17	5 ± 5	101 ± 1	34 ± 9	> 25
<b>1b</b>	R=NH <sub>2</sub> / n=3	N. A.	N. A.	101 ± 1	N. A.	> 100
<b>Functional group: Heterocyclic imine</b>						
<b>2a</b>	X=NH/Y=C/ n=2	N. A.	9 ± 7	101 ± 2	22 ± 13	> 25
<b>2b</b>	X=O/Y=C/ n=2	N. A.	5 ± 0	17 ± 12	N. A.	> 25
<b>2c</b>	X=S/Y=C/ n=2	N. A.	1 ± 3	8 ± 5	N. A.	> 25
<b>2d</b>	X=NH/Y=N/ n=2	N. A.	2 ± 1	101 ± 1	25 ± 4	> 25
<b>2e</b>	X=NH/Y=C/ n=3	N. A.	N. A.	101 ± 1	N. A.	> 100
<b>2f</b>	X=O/Y=C/ n=3	N. A.	20 ± 21	101 ± 1	N. A.	> 50
<b>2g</b>	X=S/Y=C/ n=3	N. A.	N. A.	101 ± 1	N. A.	> 50
<b>2h</b>	X=NH/Y=N/ n=3	6 ± 13	23 ± 16	99 ± 2	N. A.	> 50
<b>2i</b>	X=NH/Y=C/ n=4	4 ± 12	1 ± 12	53 ± 5	N. A.	> 50
<b>2j</b>	X=O/Y=C/ n=4	N. A.	19 ± 4	26 ± 10	N. A.	> 50
<b>2k</b>	X=S/Y=C/ n=4	N. A.	27 ± 3	94 ± 2	N. A.	> 50
<b>2l</b>	X=NH/Y=N/ n=4	N. A.	24 ± 12	24 ± 3	N. A.	> 100
<b>Functional group: Heterocyclic amine</b>						
<b>3f</b>	X=O/Y=C/N=3	N. A.	13 ± 1	99 ± 1	17 ± 15	> 25
<b>3g</b>	X=S/Y=C/N=3	N. A.	9 ± 4	96 ± 3	25 ± 10	> 50
<b>Functional group: Guanidine</b>						
<b>4a</b>	R=H/ n=2	N. A.	4 ± 10	100 ± 1	45 ± 9	> 25
<b>4b</b>	R=H/ n=3	N. A.	22 ± 8	101 ± 1	N. A.	> 50
<b>4c</b>	R=H/ n=4	N. A.	10 ± 2	N. A.	N. A.	> 100
<b>Functional group: Ureas</b>						
<b>5a</b>	R=H/ n=2	N. A.	14 ± 5	N. A.	N. A.	> 25
<b>5b</b>	R=H/ n=3	N. A.	27 ± 2	15 ± 16	3 ± 8	> 100

5c	R=H/ n=4	N. A.	16 ± 5	75 ± 12	N. A.	> 100
5d	R=4-NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub> / n=2	N. A.	11 ± 17	37 ± 8	N. A.	> 25
5e	R=4-NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub> / n=3	N. A.	N. A.	38 ± 6	26 ± 1	> 25
5f	R=4-NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub> / n=4	N. A.	10 ± 10	94 ± 4	N. A.	> 10
5g	R=CH <sub>2</sub> Ph/ n=2	N. A.	30 ± 6	5 ± 4	N. A.	> 25
5h	R=CH <sub>2</sub> Ph/ n=3	N. A.	16 ± 0	26 ± 6	21 ± 17	> 10
5i	R=CH <sub>2</sub> Ph/ n=4	N. A.	12 ± 12	74 ± 2	5 ± 8	> 10
5j	R=(CH <sub>2</sub> ) <sub>3</sub> CH <sub>3</sub> / n=2	N. A.	14 ± 1	18 ± 10	N. A.	> 50
5k	R=(CH <sub>2</sub> ) <sub>3</sub> CH <sub>3</sub> / n=3	N. A.	17 ± 14	18 ± 3	20 ± 12	> 25
5l	R=(CH <sub>2</sub> ) <sub>3</sub> CH <sub>3</sub> / n=4	N. A.	12 ± 12	96 ± 2	81 ± 8	> 10
<b>Functional group: 1,2,3-Triazole</b>						
7e	n=2	6 ± 16	13 ± 2	12 ± 4	6 ± 12	> 100
7d	n=3	N. A.	11 ± 4	16 ± 6	N. A.	> 50
7f	n=4	17 ± 2	N. A.	90 ± 3	22 ± 5	> 100

Results show means activities of at least three independent assays. NOAEL= No-Observed Adverse Effect Level (MTT assay in PMA-differentiated THP-1 cells); N. A. = no activity.

## FUNDING SOURCES AND ACKNOWLEDGEMENTS

The research leading to these results has received funding from the European Community's Seventh Framework Programme under grant agreements No.602773 (Project KINDRED). L.G. was supported by the Fundação para a Ciência e Tecnologia through grant SFRH/BD/81604/2011.

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[8] Compound 2h: white solid (84.7 %); m.p.: 212-213 °C;  $\delta_H$  (400 MHz, DMSO) 1.97-2.04 (m, 2H), 3.65 (t,  $J$ = 6.8 Hz, 2H), 4.11 (t,  $J$ = 7.6 Hz, 2H), 7.03 (s, 1H), 7.14 (s, 1H), 7.83 (t,  $J$ = 7.6 Hz, 2H), 8.19 (s, 1H), 8.41 (d,  $J$ = 8.4 Hz, 2H), 8.45 (d,  $J$ = 7.2 Hz, 2H), 12.4 (br s, 1H) ppm. Compound 2j: brown solid (45.4 %); m.p.: 110-111 °C;  $\delta_H$  (400 MHz, CDCl<sub>3</sub>) 1.82-1.87 (m, 4H), 3.68 (t,  $J$ = 6.8 Hz, 2H), 4.25 (t,  $J$ = 7.2 Hz, 2H), 6.47 (dd,  $J$ = 3.2, 1.6 Hz, 1H), 6.79 (br s, 1H), 7.51 (s, 1H), 7.76 (dd,  $J$ = 8.4, 7.2 Hz, 2H), 8.12 (s, 1H), 8.21 (dd,  $J$ = 8.4, 1.2 Hz, 1H), 8.60 (dd,  $J$ = 7.2, 1.2 Hz, 2H) ppm. Compound 2l: white solid (92.3 %); m.p.: 225-226 °C;  $\delta_H$  (400 MHz, DMSO) 1.68-1.69 (m, 4H), 3.59 (t,  $J$ = 4.8 Hz, 2H), 4.08 (t,  $J$ = 6.4 Hz, 2H), 7.02 (s, 1H), 7.16 (s, 1H), 7.84 (dd,  $J$ = 8.0, 7.2 Hz, 2H), 8.15 (s, 1H), 8.42 (dd,  $J$ = 8.4, 0.8 Hz, 2H), 8.46 (dd,  $J$ = 7.2, 1.2 Hz, 2H), 12.4 (br s, 1H) ppm.

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## Supplemental Data

Compound **2h**: white solid (84.7 %); m.p.: 212-213 °C;  $\delta_H$  (400 MHz, DMSO) 1.97-2.04 (m, 2H), 3.65 (t,  $J$ = 6.8 Hz, 2H), 4.11 (t,  $J$ = 7.6 Hz, 2H), 7.03 (s, 1H), 7.14 (s, 1H), 7.83 (t,  $J$ = 7.6 Hz, 2H), 8.19 (s, 1H), 8.41 (d,  $J$ = 8.4 Hz, 2H), 8.45 (d,  $J$ = 7.2 Hz, 2H), 12.4 (br s, 1H) ppm. Compound **2j**: brown solid (45.4 %); m.p.: 110-111 °C;  $\delta_H$  (400 MHz, CDCl<sub>3</sub>) 1.82-1.87 (m, 4H), 3.68 (t,  $J$ = 6.8 Hz, 2H), 4.25 (t,  $J$ = 7.2 Hz, 2H), 6.47 (dd,  $J$ = 3.2, 1.6 Hz, 1H), 6.79 (br s, 1H), 7.51 (s, 1H), 7.76 (dd,  $J$ = 8.4, 7.2 Hz, 2H), 8.12 (s, 1H), 8.21 (dd,  $J$ = 8.4, 1.2 Hz, 1H), 8.60 (dd,  $J$ = 7.2, 1.2 Hz, 2H) ppm. Compound **2l**: white solid (92.3 %); m.p.: 225-226 °C;  $\delta_H$  (400 MHz, DMSO) 1.68-1.69 (m, 4H), 3.59 (t,  $J$ = 4.8 Hz, 2H), 4.08 (t,  $J$ = 6.4 Hz, 2H), 7.02 (s, 1H), 7.16 (s, 1H), 7.84 (dd,  $J$ = 8.0, 7.2 Hz, 2H), 8.15 (s, 1H), 8.42 (dd,  $J$ = 8.4, 0.8 Hz, 2H), 8.46 (dd,  $J$ = 7.2, 1.2 Hz, 2H), 12.4 (br s, 1H) ppm.

## 4.2.2. Target-based drug discovery

### 4.2.2.1. Inhibitors of *Trypanosoma cruzi* Sir2 related protein 1 as potential drugs against Chagas disease

Luís Gaspar, David M. Costa, Begoña Pérez-Cabezas, Joana Tavares, Nuno Santarém, Alain Pruvost, Isbaal Ramos, Ian K. Pemberton, Paul K. Thoo-Lin, Celine Ronin, Paola Ciapetti and Anabela Cordeiro-da-Silva

*Manuscript in preparation*

#### Objectives of the study:

In this work, it was aimed to characterize the biochemical activity of *T. cruzi* Sir2rp1 (TcSir2rp1) and the determination of its kinetic constants. In addition, it was analyzed if the enzyme would have druggable properties as assessed by inhibition by nicotinamide and by a family of compounds known to inhibit trypanosomal sirtuins, the BNIP derivatives. Finally, the activity of the compounds against the whole parasite in an *in vitro* assay as well as an *in vivo* model of disease was also determined.

#### Main results:

- *T. cruzi* Sir2rp1 is a canonical sirtuin with NAD<sup>+</sup>-dependent deacetylase activity.
- The enzyme showed to be druggable, as demonstrated by the inhibition by the small molecule compound, nicotinamide.
- Some BNIP derivatives are active against TcSir2rp1.
- The BNIP derivatives are also active against intracellular amastigotes, albeit with low selectivity for a majority of the compounds.

→ The most selective compound, BNIPSpd does not present any *in vivo* activity.

### **Conclusion:**

*T. cruzi* Sir2rp1 is, from a biochemical standpoint, a potential target against the parasite. Its deacetylase activity is readily inhibited not only by classic inhibitors, but also by experimental compounds like the trypanosomal sirtuin inhibitors BNIP derivatives. The *in vitro* activity of these inhibitors highlights the possibility of the synthesis of more potent and selective compounds.

## **Inhibitors of *Trypanosoma cruzi* Sir2 related protein 1 as potential drugs against Chagas disease**

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## ABSTRACT

Chagas disease remains one of the most neglected diseases in the world and is the most important parasitic disease in Latin America. The characteristic chronic manifestation of chagasic cardiomyopathy is a region's leading cause of heart-related illness, causing significant mortality and morbidity. Due to the limited available therapeutic options, new drugs are an urgent need to control the disease. Sirtuins also called Silent information regulator 2 (Sir2) proteins have long been suggested as interesting targets to treat different diseases, including parasitic infections. Recent studies on *Trypanosoma cruzi* sirtuins have hinted at the possibility to exploit this enzyme as a possible drug target. In the present work, the *T. cruzi* Sir2 related protein 1 (TcSir2rp1) is biochemically characterized for its deacetylase activity and its inhibition by the classic sirtuins inhibitor nicotinamide as well as by bisnaphthalimidopropyl (BNIP) derivatives, a class of parasitic sirtuins inhibitors. BNIPs ability to inhibit TcSir2rp1 and antiparasitic activity against *T. cruzi* amastigotes *in vitro* were investigated. BNIPSpd was found to be the most potent inhibitor of TcSir2rp1. Moreover, this compound showed an anti-parasitic activity similar to the reference drug benznidazole and the highest selectivity index among the compounds tested. Unfortunately, BNIPSpd failed to treat a preclinical mice model of Chagas disease possibly due to its pharmacokinetic profile. Medicinal chemistry modifications of the compound, as well as alternative formulations may improve activity and pharmacokinetics. In conclusion, the search for TcSir2rp1 specific inhibitors may represent a valuable strategy for drug discovery against *T. cruzi*.



## 1. Introduction

Chagas disease, caused by the protozoan *Trypanosoma cruzi*, remains one of the most prevalent and neglected diseases in the world [1] and is the parasitic infirmity with the highest socio-economic burden in Latin America [2, 3]. While the vast majority of the newly acquired cases are asymptomatic, about 20 to 30% of the cases lead to the development, usually decades later, of the characteristic chronic chagasic cardiomyopathy that is the leading cause of non-ischemic heart disease in the continent [4, 5].

The parasite has a complex life cycle, with two hosts and four different stages: the replicative epimastigotes and the infective, non-replicative metacyclic trypomastigotes in the triatomine insect vector and the intracellular, replicative amastigote and the infective, non-replicative bloodstream trypomastigote in the mammalian host. This host can be the Humans or another of the more than one hundred mammalian species that act as natural reservoirs [6]. Although vectorial transmission has been greatly reduced in recent years, other transmission mechanisms like congenital, oral and blood and organ transfusion transmission still contribute to about 40,000 new cases and 12,000 deaths every year [7]. What used to be a local concern, has become a worldwide problem due to human migrations to other parts of the world like North America, Europe and Japan [8].

There is no available vaccine for Chagas disease, and the therapy relies on two drugs introduced more 40 years ago: benznidazole and nifurtimox. These drugs are far from ideal because of the many toxic effects and the requirement to administer them for long periods of time [4, 9, 10]. Thus, new drugs are an urgent need. Very few drugs have populated the pipeline for Chagas disease in the last decade, with most of them acting by the same mechanism [11]. The recent failure of clinical trials for two of these drugs, posaconazole and E1224, has exposed the fragilities of the chemotherapeutic pipeline and reinforced the need for constant drug discovery efforts to find alternative therapies [12-14].

Sir2 (silent information regulator 2) or sirtuins are class III histone deacetylases that are evolutionary conserved and present across various kingdoms of life, from bacteria to humans [15, 16]. They catalyze the NAD<sup>+</sup>- dependent deacetylation of acetylated lysine residues in a polypeptide chain, according to the reaction:



Besides the deacetylated lysine, other products are formed in the reaction, like nicotinamide (an endogenous inhibitor) and 2'-O-acetyl-ADP-ribose (2'-O-AADPr), that has been suggested as a second messenger [17] and has a function in promoting the

association of the complex Sir3/Sir2/Sir4 involved in yeast gene silencing [18]. Some human sirtuins like SIRT4 and SIRT6 and the protozoan Sir2 related protein 1 (Sir2rp1) from *T. brucei* and *L. infantum* also display ADP-ribosyltransferase activity [19-22]. However, the biologic role of ADP-ribosylation by sirtuins has not been clearly demonstrated and is currently debated to be a nonspecific side reaction [23]. In addition, some sirtuins have also been characterized to perform deacylase reactions beyond deacetylation, as is the case of SIRT5 that possesses demalonylase, desuccinylase and deglutarylase activity [24, 25].

Sirtuins have been attributed many roles in different organisms, including life span regulation, cell cycle progression, gene transcription, apoptosis, DNA repair and metabolism [26-29]. The human genome, as well as other mammals' genomes, codify 7 distinct sirtuins (SIRT1-7) [30], whose proteins are distributed in different cell compartments: SIRT1, SIRT6 and SIRT7 are located in the nucleus, SIRT2 is cytoplasmic (but shuttles to the nucleus), whereas SIRT3, SIRT4 and SIRT5 are found in the mitochondria [31]. Their different localizations are related with their cellular functions, for instance: nuclear SIRT1, 6 and 7 are involved in transcription regulation [32], DNA repair [33, 34] and chromatin remodeling [35, 36], respectively; SIRT2 is a tubulin deacetylase that co-localizes with the cytoskeleton but is imported to the nucleus where it participates in cell cycle regulation [37]; and mitochondrial sirtuins SIRT3, 4 and 5, participate in fatty acid metabolism [38], amino acid metabolism [19] and the urea cycle, respectively [39].

By contrast, parasitic protozoa have less sirtuin homologues but they have also been described as “pro-life” proteins due to their importance for the normal development and functioning of these cells. For instance, in the apicomplexan *Plasmodium falciparum*, Sir2A and B are involved in antigenic variation, an essential process for immune system evasion [40, 41]. Trypanosomatids have between two to three homologues (Sir2rp1-3) with some of the members varying in their functions and subcellular localizations. *T. brucei* Sir2rp1 is the most characterized of the three enzymes, is located in the nucleus and seems to be important for the protection against DNA damage [21, 42]. TbSir2rp2 and TbSir2rp3 are localized in the mitochondria and little is known about their function, except that they are all not essential for parasite survival [42]; *Leishmania* species also encode for three enzymes, but most studies are focused on Sir2rp1, that has a cytoplasmic localization. LiSir2rp1 from *L. infantum* is required for the normal replication of amastigotes [43]. *T. cruzi* genomes sequenced so far only revealed two homologues, Sir2rp1 and Sir2rp3. Two recent reports describe their localization in the cytoplasm and the mitochondria, respectively [44, 45]. Overexpression of TcSir2rp1 and TcSir2rp3 revealed to interfere with epimastigotes growth, differentiation into metacyclic trypomastigotes, infectivity of host cells and intracellular amastigotes replication [44, 45]. It should be noted that some contradictory

findings have been reported by both research groups, differences that are likely related with the strategy of overexpression (constitutive [44] versus inducible [45]). A clearer role of the participation of Sir2rp1 and Sir2rp3 in *T. cruzi* life cycle and infectivity should be elucidated by gene knockout studies.

In this work, we characterized for the first time the enzymatic activity of TcSir2rp1 and search for specific inhibitors among BNIPs derivatives with anti-trypanosomal activity [46]. We demonstrate that TcSir2rp1 is a NAD<sup>+</sup>-dependent deacetylase that is inhibited by classic inhibitors as well as by some BNIP derivatives. Furthermore, we demonstrate that these inhibitors are also active against *T. cruzi* amastigotes, suggesting that TcSir2rp1 may be a viable drug target to explore for the chemotherapeutic control of Chagas disease. In addition, the *in vivo* activity of the most potent inhibitor and selective compound towards *T. cruzi* was evaluated by whole mice bioluminescent imaging.

## 2. Materials and methods

### 2.1. Ethics statement

All experiments involving animals were carried out in accordance with the IBMC/INEB Animal Ethics Committees and the Portuguese National Authorities for Animal Health guidelines, according to the statements on the directive 2010/63/EU of the European Parliament and of the Council.

### 2.2. Cells and Parasites

Mouse myoblast C2C12 cell line, green monkey kidney epithelial Vero cells were cultured with high glucose DMEM supplemented with 10% FBS, 25 mM HEPES, 2 mM glutamine, 100 U/mL penicillin, 100 U/mL streptomycin and maintained in a humid 5% CO<sub>2</sub> atmosphere at 37°C.

*T. cruzi* wt and Luc<sup>+</sup> Y strain trypomastigotes were maintained by *in vitro* infection of a monolayer of Vero cells and harvested by collection of the supernatant after 5 to 7 days. The trypomastigotes were used for the *in vitro* screening and to re-infect new monolayers for up to 10 cycles, after which point the cultures were rejected and restarted from a new frozen stock. Luminescent Y strain parasites were obtained by transfection as previously described [47]. Briefly, 10 µg of the overexpression plasmid pTREX [48], containing the sequence for firefly luciferase, were transfected into logarithmic Y strain epimastigotes using AMAXA Nucleofector II device (program V-33) with the Human T-cell nucleofector kit

(Lonza). Twenty-four hours after transfection, 75 µg/mL of G418 was added to the culture and parasites were selected for 6 weeks. Epimastigotes cultures were allowed to enrich for metacyclic trypomastigotes for 3 weeks, after which the parasites were used to infect monolayers of Vero cells in the presence of G418. The Luc<sup>+</sup> tissue culture trypomastigotes released were maintained in culture by cycles of re-infection of Vero cells.

### 2.3. Cloning, expression and purification of TcSir2rp1

The full coding sequence of TcSir2rp1 (accession number: XP\_818420.1) was PCR amplified from *T. cruzi*, CL Brener strain genomic DNA using the primers 5'-CCATGGGAATGAATCAAGATAACGCCAACTTT-3' and 5'-CTCGAGTTTTCGGTCTGTCTGTGTGTACATG-3'. The gene was cloned into pET-28a (Novagen) using the NcoI and XhoI restriction sites, resulting in a C-terminal 6xHis tagged fusion protein. The plasmid was transformed into BL21 (DE3) *E. coli* for inducible expression. Bacteria were grown at 37°C to an O.D. of 0.6 prior to overnight induction with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 18°C. Cells were then pelleted, resuspended in buffer containing 500 mM NaCl, 20 mM Tris, pH 7.6 and disrupted by sonication. The lysate was cleared by centrifugation, applied to a Ni<sup>2+</sup>-NTA resin column and eluted with solutions of increasing concentration of imidazole. The purest fractions were re-purified a second time by the same technique to remove remaining contaminants as visible by SDS-PAGE Coomassie blue staining. The purified protein solution was passed through a PD-10 desalting column (GE Healthcare) and the buffer exchanged to PBS. Protein concentration was determined by DC Protein Assay (Bio-Rad) and aliquots were stored at -70°C. Protein was Western blotted using a rabbit polyclonal anti-HisTag antibody in a dilution of 1:1000.

*T. brucei* Sir2rp1 tagged with a hexahistidine tail has been previously expressed and characterized [49]. In this study, a pT7 expression vector containing the sequence for TbSir2rp1 (accession number: AAX70528.1) was used. Due to poor protein solubility, the plasmid was transformed into pLys BL21 (DE3) *E. coli* instead. Bacteria logarithmic grown at 37°C to an O.D. of 0.6 were subject to a physicochemical shock by incubation with 2% ethanol in a water/ice bath for 30 minutes. Expression induction with IPTG at 0.1 mM was immediately followed at 18°C for 12 hours. Purification by affinity chromatography, buffer exchange, protein quantification and storage was performed similarly to TcSir2rp1.

*L. infantum* Sir2rp1 was expressed and purified as previously described [22].

## 2.4. Deacetylation assays

The deacetylase activity was measured using the commercial SIRT1/Sir2 Deacetylase Fluorometric Assay Kit from CycLex (Japan) according to the manufacturer's recommendations. Briefly, an acetylated substrate peptide with an N-terminal fluorophore and a C-terminal fluorescence quencher is deacetylated by a deacetylation enzyme (e.g. sirtuin) and becomes the substrate for a lysylendopeptidase that breaks the substrate and emits fluorescence. Fluorescence was measured in a fluorimetric plate reader (Synergy HT, BioTek) with excitation and emission wavelengths set at 340 and 440 nm, respectively, every 30 seconds for 1 hour. The slope of the linear part of the reaction was calculated and used as readout of enzyme activity.

Kinetic constants were determined with 0.5 µg of TcSir2rp1. For the determination of the peptide substrate constants, NAD<sup>+</sup> concentration was fixed at an excess of 2000 µM while the peptide substrate concentration was varied between 0.63 and 40 µM. For the NAD<sup>+</sup> constants, the peptide substrate concentration was kept in excess at 40 µM while NAD<sup>+</sup> concentration was varied from 15.63 to 2000 µM. Initial velocities were measured between 4 and 6 minutes where steady state conditions were assumed. Data was analyzed with Graphpad Prism version 6.0 software using the built-in enzyme kinetics, Michaelis-Menten equation regression for  $K_m$  and  $v_{max}$  determinations.  $k_{cat}$  was calculated using the formula:

$$K_{cat} = \frac{V_{max}}{[E]_i}$$

## 2.5. Synthesis of BNIP derivatives

The following compounds were synthesized as previously reported **1-11** [46,50], **6b** and **9a** [51], and **6c** [52]. Compounds 1a-c, 6a, 7a 12, 13 were newly synthesized according to the conditions described below. All starting materials were purchased from Sigma-Aldrich, were of research-grade quality and were used without further purification. Thin Layer Chromatography (TLC) was performed on silica gel 60 F254 aluminum plates (Merck) in dichloromethane/methanol (95:5). NMR was recorded on a Bruker 400 Ultrashield spectrometer at 400.1 MHz for <sup>1</sup>H and 100.6 MHz for <sup>13</sup>C.

### 2.5.1. Synthesis of BNIPDmPP, **12**

In a round bottomed flask, 1, 4 bis(3-aminopropyl) piperazine (0.3g, 1.498 mmol) and 1, 8-Naphthalic anhydride (3.011 mmol, 0.539g, 2.01 x excess) was dissolved in DMF (7 mL) followed by the addition of DBU (1 mL). The solution was stirred for 2 hours at 70°C

and was monitored using TLC. When the reaction was complete, the solution was poured into 50 mL of icy water and stirred with a glass rod to form a precipitate. The precipitate was filtered off, washed thoroughly with water and dried under vacuum at 50°C for 2 hours. The product, Bisnaphthalimidopropyl-piperazine (87% yield), required no further purification for the next step. Bisnaphthalimidopropyl-piperazine was then methylated. In a round bottomed flask, bisnaphthalimidopropyl-piperazine (0.25 g, 0.4464 mmol) was dissolved in 25 mL of anhydrous DCM followed by the addition of methyl-iodide (2 mL). The solution was boiled under reflux overnight to yield a precipitate which was filtered off and washed with 20 mL of anhydrous DCM. The methyl-iodide quaternary salt of bisnaphthalimidopropyl-piperazine (BNIPDmPP) (69% yield) was dried under *vacuo* at 50°C overnight.

<sup>1</sup>H (DMSO-d<sub>6</sub>): δ 8.46-8.38, 7.85-7.79 (aromatic protons), 4.08 (triplet, CH<sub>2</sub>), 4.00 (triplet, CH<sub>2</sub>), 3.46-3.41, 3.34-3.26 (multiplet, 2 x CH<sub>2</sub>), 2.93 (singlet, 3H, CH<sub>3</sub>), 2.76-2.72 (2H, CH<sub>2</sub>), 2.57-2.54 (2H, CH<sub>2</sub>), 2.09-2.05 (multiplet, 2H, CH<sub>2</sub>), 1.74-1.71 (multiplet, 2H, CH<sub>2</sub>).

HRMS (ESI): calculated for C<sub>35</sub>H<sub>35</sub>N<sub>4</sub>O<sub>4</sub> I, 702.1703, found: 575.2653 [M-I]<sup>+</sup>.

### 2.5.2. Synthesis of BCNIPP, **13**

In a round bottomed flask, (0.596 g, 3.01 mmol) of 4-chloro-1,8-naphthalic anhydride was dissolved in 7 ml of DMF. 0.3g (1.498mmol) of bis(3-aminopropyl)piperazine were then added to the solution, followed by 0.7 ml of DBU. The solution was left to stir at 70°C for 48 hours. When complete, the reaction solution was poured in cold water (100ml) while stirring to form a precipitate as the product. This precipitate was then filtered with a Buchner funnel and washed up with water. The solid obtained was dried under *vacuo*, to yield BCNIPP (68% yield).

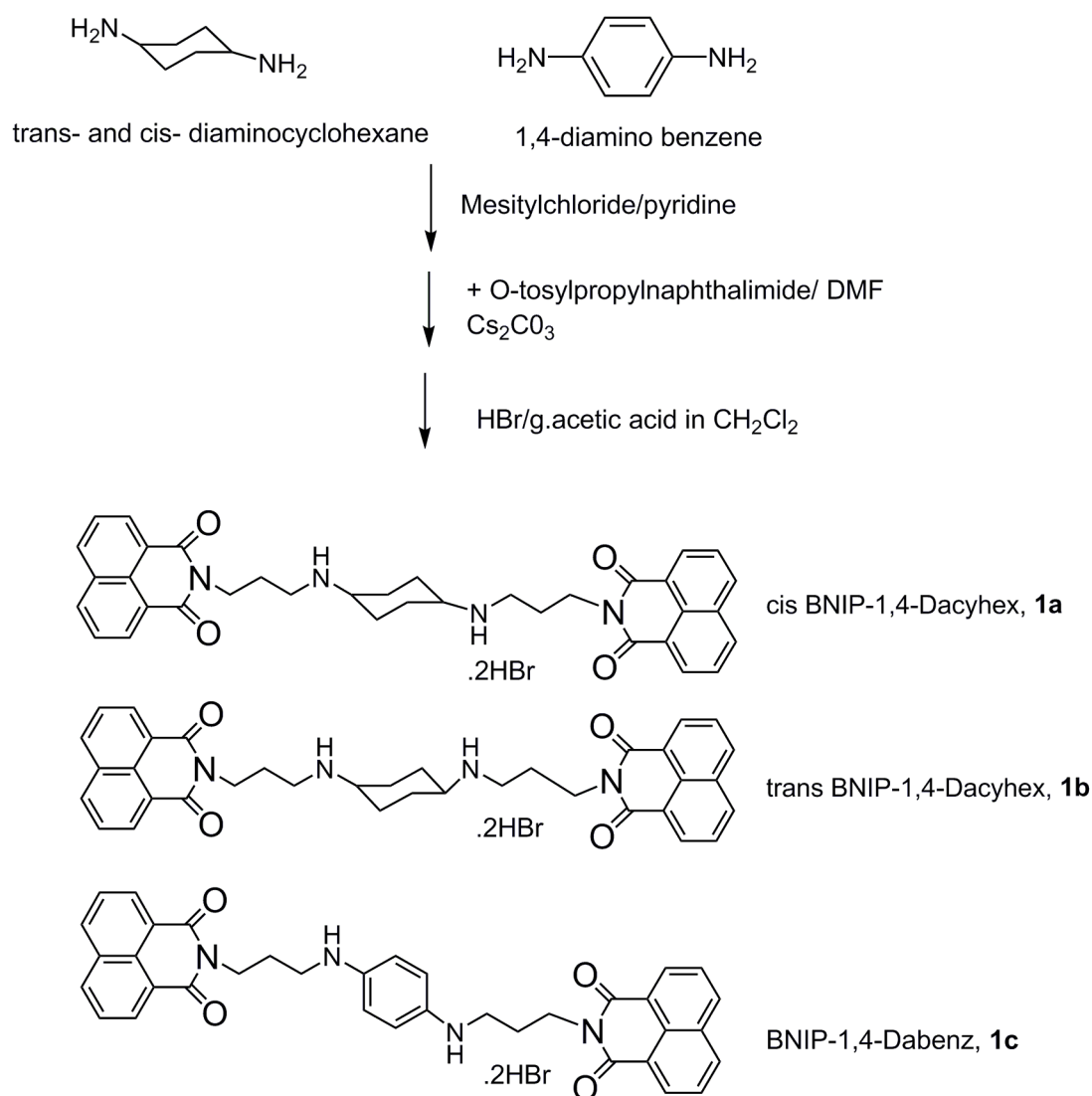
<sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 8.56-8.40; 7.76-7.73 (naphthalimido ring protons), 4.12 (triplet, CH<sub>2</sub>-naphthalimide), 2.21 (triplet, CH<sub>2</sub> piperazine ring and CH<sub>2</sub>-N), 1.77 (pentet, CH<sub>2</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ = 163.5 (4-chloro-naphthalimide carbonyl), 138.97-121.75 (4-chloro-naphthalimide carbons), 56.07 (CH<sub>2</sub>-naphthalimide), 53.01 (piperazine CH<sub>2</sub>), 39.06 (CH<sub>2</sub>-piperazine), 24.89 (CH<sub>2</sub>).

HRMS (ESI): calculated for C<sub>34</sub>H<sub>30</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>4</sub>: 629.1644 (M+H); found: 629.1716.

### 2.5.3. Synthesis of cis BNIP-1,4-Dacychex, **1a**, trans BNIP-1,4-Dacychex, **1b**, and BNIP-1,4-Dabenz, **1c**

All the above diamines were treated with mesitylchloride in pyridine to yield the corresponding bismesityl derivatives in quantitative yields. Subsequent reaction N-alkylation with O-tosylpropylnaphthalimide [51] gave the corresponding protected

bisnaphthalimidopropyl derivatives which upon deprotection with HBr/g.acetic acid in dichloromethane [51] yielded **1a**, **1b** and **1c** (Figure 1).



**Figure 1.** Schematic representation of the synthesis of compounds: cis BNIP-1,4-Dacyhex (**1a**), trans BNIP-1,4-Dacyhex (**1b**) and BNIP-1,4-Babenz (**1c**).

**Cis BNIP-1,4-Dacyhex, 1a**

HRMS (ESI): calculated for C<sub>36</sub>H<sub>36</sub>N<sub>4</sub>O<sub>4</sub> 2HBr: 748.1261, 589.2809 ([M+H]<sup>+</sup>-2HBr), found: 589.2798 ([M+H]<sup>+</sup>-2HBr).

**Trans BNIP-1,4-Dacyhex, 1b**

After N-alkylation step, the crude was thoroughly dried in a vacuum oven. The pale brown solid was re-suspended in hot toluene. The latter was quickly filtered and washed

with hot toluene. The resulting precipitate was dried under *vacuo* and the solid obtained gave a single spot on TLC (64%) as the protected trans BNIP-1,4-Dacyhex.

The dibromide salt was obtained in quantitative yield.

$^1\text{H}$  NMR (400.1 MHz, DMSO- $d_6$ ):  $\delta$  8.70-7.60 (m, aromatic naphthalimido ring protons), 4.20 (t, 2 x  $\text{CH}_2\text{-N}$ ), 3.40 (s,  $\text{CH}_2\text{-N}$ ), 3.10 (s, NH), 2.20-2.10 (m,  $\text{CH}_2$ ), 1.50 (t, CH).  $^{13}\text{C}$  NMR (DMSO):  $\delta$  164.26 (C=O), 134.92-122.64 (Aromatic protons from the Naphthalimido rings), 55.06 (CH), 42.74 ( $\text{CH}_2$ ), 37.59 ( $\text{CH}_2$ ), 26.91 ( $\text{CH}_2$ ).

HRMS (ESI): calculated for  $\text{C}_{36}\text{H}_{36}\text{N}_4\text{O}_4 \cdot 2\text{HBr}$ : 748.1261, 589.2809 ( $[\text{M}+\text{H}]^+-2\text{HBr}$ ), found: 589.2808 ( $[\text{M}+\text{H}]^+-2\text{HBr}$ ).

#### BNIP-1,4-Dabenz, **1c**

*p*-phenylenediamine, **1**, (1 g; 9.25 mmol) was dissolved in pyridine (20 mL) and the solution was stirred. Mesithylenesulphonyl (Mts) chloride (4.25 g; 19.425 mmol) (2.1 M excess) was then added and the reaction was left stirring for 4h at room temperature. After this time, pyridine was removed, the remaining liquid was poured in icy water (200 mL) and a precipitate was formed. The solution was filtered off and washed with water several times. The solid was dried in the vacuum oven overnight to afford N,N-bismesityldiaminobenzene in 93.3% yield.

$^1\text{H}$  NMR (DMSO- $d_6$ ):  $\delta$  9.9 (2H, NH), 6.9 (4H, s, Aromatic H), 6.8 (4H, s, aromatic H), 2.4 (12H, s, 4 x  $\text{CH}_3$ ), 2.2 (6H, s, 2 x  $\text{CH}_3$ ).  $^{13}\text{C}$  NMR (DMSO- $d_6$ ):  $\delta$  142.3-122.0 (aromatic carbons), 22.8 ( $\text{CH}_2$ ), 20.8 ( $\text{CH}_2$ ).

N, N-bismesityldiaminobenzene (0.1 g) was dissolved in DMF (2.5 mL), followed by the addition of O-tosylpropylnaphthalimide (2.4 M excess) immediately followed by caesium carbonate (5 M excess). The solution was left stirring overnight at 70 °C. Reaction completion was monitored by thin layer chromatography. The solution was poured into icy water (80 mL) to form a precipitate. Precipitate was filtered off, washed thoroughly with water and dried in *vacuo*. Fully protected N,N-bismesityldiaminobenzene:  $^1\text{H}$  NMR (400.1 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.50-7.70 (m, aromatic protons), 7.30, 6.80 (d, mesityl aromatic protons), 7.20 (s, diaminobenzene aromatic protons), 4.2 (t,  $\text{CH}_2\text{-N}$ ), 3.8 (t,  $\text{CH}_2\text{-N}$ ), 2.4 (s, Ar- $\text{CH}_3$ ), 2.2 (s, Ar- $\text{CH}_3$ ), 1.9 ppm (t,  $\text{CH}_2$ ).  $^{13}\text{C}$  (100.6 MHz,  $\text{CDCl}_3$ ):  $\delta$  164.00 (C=O), 134.00-122.00 (aromatic carbons), 143.00-132.00 (Mesityl aromatic carbons), 140.00-138.00 (diaminobenzene aromatic carbons), 48.00, 38.00, 27.00 (3 x  $\text{CH}_2$ ), 23.00, 21.00 ppm (Mesityl- $\text{CH}_3$ ).

Protected BNIP-1,4-Dabenz (0.1 g; 0.106 mmol) was dissolved in DCM (5 mL) with heating up and stirring. HBr/ $\text{CH}_3\text{COOH}$  solution (385  $\mu\text{L}$ ) was added and solution was left in the fume cupboard at room temperature four days to form very fine precipitate which was



centrifuged, washed with DCM and ether and centrifuged after each wash to give the final product BNIP-1,4-Dabenz **1c** (23.% yield).  $^1\text{H}$  (DMSO- $d_6$ ): 8.51-8.40, 7.89-7.84, 7.19-6.90 (Aromatic protons from the naphthalimido and benzene rings, 4.15-4.10 (4H, 2xCH<sub>2</sub>), 3.22-3.19 (4H, 2xCH<sub>2</sub>), 1.99-1.92 (4H, 2xCH<sub>2</sub>).  $^{13}\text{C}$  NMR (DMSO- $d_6$ ):  $\delta$  164.10 (C=O), 134.80-122.60 (aromatic carbons), 55.60 (CH<sub>2</sub>), 37.90 (CH<sub>2</sub>).

LRMS for C<sub>36</sub>H<sub>30</sub>N<sub>4</sub>O<sub>4</sub> was 742.09, 582.23 [M-2HBr]<sup>+</sup>, found: 582.20 [M-2HBr]<sup>+</sup>, 583.20 ([M+H]<sup>+</sup>) and 605.0 m/z ([M+Na]<sup>+</sup>).

#### 2.5.4. Synthesis of BNIPDPP salt, **6a**

In a round bottom flask (50 cm<sup>3</sup>), 1,3-bis-(4-piperidyl)propane (0.25 g, 1.19x10<sup>-3</sup> mol,) and toluenesulfonyloxypropylnaphtalamide (0.98 g, 2.39x10<sup>-3</sup> mol) were dissolved in THF(6 mL). The reaction was refluxed at 50°C for 15 minutes followed by the addition of caesium carbonate (1 g, 3.069x10<sup>-3</sup> mol), the reaction was left to stir overnight at 50°C. The resulting solution was poured into icy water (100 mL). A precipitate was formed and after vacuum filtration, the product was dried in a vacuum oven at 45°C overnight. The crude product (base of BNIPDPP) was recrystallized from ethanol (64.8%).

$^1\text{H}$ -NMR (CDCl<sub>3</sub>):  $\delta$ H 8.52– 8.50 (2H, CH aromatic protons), 8.15– 8.11 (2H, CH aromatic protons), 7.70–7.65 (2H, CH aromatic protons), 4.17– 4.14 (2, CH<sub>2</sub> protons), 2.84 – 2.82 (2H, CH<sub>2</sub> protons), 2.41 – 2.37 (2H, CH<sub>2</sub> protons), 1.91-1.83 (2H, CH<sub>2</sub> protons), 1.79-1.74 (2H, CH<sub>2</sub> protons), 1.51-1.48 (2H, CH<sub>2</sub> protons), 1.71-1.09 (H, CH protons) ppm.

In a round bottom flask (50 cm<sup>3</sup>), the free base BNIPDPP (1 g, 1.459x10<sup>-3</sup> mol) was dissolved in DCM (20 mL) and HBr/CH<sub>3</sub>CO<sub>2</sub>H (2 mL) was added slowly. The reaction was stirred for 2 hours at room temperature to yield a precipitate. The latter was filtered off by and washed with DCM (30 mL) and ether (10 mL). The BNIPDPP salt was dried under *vacuo* at 45°C for 2 hours (yield 72.3%).

$^1\text{H}$ -NMR (CDCl<sub>3</sub>):  $\delta$ H 8.53 – 8.51 (2H, CH aromatic protons), 8.14 – 8.12 (2H, CH aromatic protons), 7.70 – 7.66 (2H, CH aromatic protons), 4.69 (2H, CH<sub>2</sub> protons), 4.17 – 4.14 (2H, CH<sub>2</sub> protons), 2.84 – 2.82 (2H, CH<sub>2</sub> protons), 2.41 – 2.37 (2H, CH<sub>2</sub> protons), 1.91-1.83 (2H, CH<sub>2</sub> protons), 1.79-1.74 (2H, CH<sub>2</sub> protons), 1.51-1.48 (2H, CH<sub>2</sub> protons), 1.71-1.09 (H, CH proton) ppm.

HRMS (ESI): calculated for C<sub>43</sub>H<sub>48</sub>N<sub>4</sub>O<sub>4</sub> [M+1]<sup>+</sup> - 2Br: 685.3748, found: 685.3736.

#### 2.5.5. Synthesis of BNIPDapp, **7a**

Synthesis of N,N-dimesityl-aminopropylpiperazine. In a round bottomed flask, 1 g of 1,4 bis(3-aminopropyl)piperazine (0.0499 mol) was dissolved in 10 mL of anhydrous

pyridine. The solution was stirred at 0°C (on ice) then mesitylenesulfonylchloride (Mts-Cl) (2.19 g, 0.01003 mol, 2.01 x excess) was added slowly, over 15 minutes. The reaction was stirred for 1 hour at 0°C (on ice) and monitored using TLC. When complete, the solution was poured into 50 mL of icy water while stirring with a glass rod and left to settle for 15 minutes to form a precipitate. The latter was filtered off, washed thoroughly with water and dried under *vacuo*, followed by recrystallisation from ethanol (36%). In a round bottomed flask, N,N-dimesityl-aminopropylpiperazine (0.3 g, 0.5357 mmol) and O-tosylpropylphthalimide (0.46 g, 1.125 mmol, 2.01 x excess) was dissolved in 6 mL of DMF. Once fully dissolved cesium carbonate (0.873 g, 2.6785 mmol) was added slowly. The solution was stirred overnight at 60°C. When complete, the solution was poured into 50 mL of icy water and stirred with a glass rod to yield a precipitate. The latter was filtered off, washed thoroughly with water and then dried under vacuum at 50°C. The crude product was recrystallized from ethanol to give BNIP-Di-Mts-aminopropylpiperazine (49% yield). In a round bottomed flask, 0.23 g of BNIP-Di-Mts-aminopropylpiperazine (0.222 mmol) was dissolved in 10 mL of anhydrous dichloromethane (DCM) followed by the addition of 1 mL of hydrobromic acid in glacial acetic acid (HBr/gCH<sub>3</sub>CO<sub>2</sub>H). The solution was left stirring for 24 hours at room temperature and monitored using TLC. When complete, the precipitate formed was filtered off and washed with 20 mL of anhydrous DCM and 5 mL of ether. The dihydro-bromic salt of bisnaphthalimidopropyl-diamino-propylpiperazine (BNIPDapp) (75% yield).

BNIPDapp: <sup>1</sup>H NMR (DMSO): δ 8.5-7.5 (aromatic protons), 4.1 (CH<sub>2</sub>-Naphth), 2.6 (CH<sub>2</sub>-NH, in chain), 2.5 (CH<sub>2</sub>-N, from ring), 2.0 (NH) and 1.5 (CH<sub>2</sub>). <sup>13</sup>C NMR (DMSO): δ 165.0 (C=O), 140.0–120.0 (aromatic carbons), 70.0 (CH<sub>2</sub>, from ring), 20.0-50.0 (CH<sub>2</sub>, from chain). HRMS (ESI): calculated for C<sub>40</sub>H<sub>48</sub>N<sub>6</sub>O<sub>4</sub>Br<sub>2</sub>, 833.1846 [M-2H-Br]<sup>+</sup>, found: 833.1844 [M-2H-Br]<sup>+</sup>

## 2.6. Screening of enzyme inhibitors

A class of bisnaphthalimidopropyl (BNIPs) derivatives previously described [46] as well as the newly synthesized derivatives were evaluated as potential TcSir2rp1 inhibitors. The enzymatic reactions were performed in the presence of the various inhibitors, 200 μM NAD<sup>+</sup> and 10 μM of peptide substrate. The inhibition is expressed in percentage and was calculated as the ratio of velocity for the linear portion of the reaction, normalized with a no drug control and a reference drug control (nicotinamide at 2 mM).

## 2.7. Anti-*Trypanosoma cruzi* activity assays

For the evaluation of the activity against *T. cruzi* amastigotes an assay using high-content screening (HCS) was designed. In summary, 100  $\mu\text{L}$  of host C2C12 cells were seeded in clear bottom black plates at a density of  $2.5 \times 10^4$  cells/mL ( $2.5 \times 10^3$  cells/well). After 24 hours, 50  $\mu\text{L}$  of Y strain tissue culture-derived trypomastigotes were used to infect the host cells at a density of  $7.5 \times 10^5$ /mL ( $3.75 \times 10^4$  parasites/well). The parasites were allowed to infect for a 24 hour period, after which compounds were added in a volume of 50  $\mu\text{L}$ . Final concentration of DMSO in the assay did not exceeded 0.5%. After 72 hours of compound incubation, the wells were fixed with 4% paraformaldehyde for 15 to 30 minutes. The plates were then washed once with deionized water and stained with a solution of DAPI (3  $\mu\text{M}$ ) for 1 hour. Plates were imaged in an INCell 2000 (GE Healthcare) high-content analyzer by taking 16 pictures per well at 20X objective amplification. Images were analyzed with INCell Developer software (GE Healthcare) using the segmentation of host cell nuclei and parasite kinetoplast DNA (kDNA). The measurement output used was the average number of parasites per cell calculated by the ratio of host cell nuclei/parasite kDNA.

## 2.8. Evaluation of BNIPSpd potential toxicity *in vitro*

To evaluate *in vitro* toxicity towards mammalian cells (hepatocytes, neurons and MDCK cells, a renal cell line from dogs), all the compounds were assayed with MTT [53]. Additionally, for cardiotoxicity evaluation another methodology was preferred as toxic effects in cardiomyocytes could be affecting their function before being lethal. Therefore, we performed a test that determines the function of the hERG channel in response to the test compound. The hERG assay was performed according to the manufacturer's protocol Predictor hERG Fluorescence Polarization Assay (Invitrogen). Briefly, inhibition of hERG channel was analyzed by competition of BNIPSpd and a high-affinity red fluorescent hERG channel ligand. BNIPSpd was mixed with the ligand and membranes containing hERG channel, incubated for 2 hours at room temperature and the fluorescence polarization was measured using 530 nm/590 nm ex/em filters using the Synergy 2 plate reader (Biotek). A set of *in vitro* assays were also performed in primary cells (hepatocytes and neurons) to evaluate BNIPSpd potential toxicity: (a) host cell nuclei counting after HOECHST staining, (b) viability by measuring the metabolization of WST-8 probe, (c) apoptosis induction through caspase 3/7 activation, (d) evaluation of mitochondrial dysfunction by measuring the membrane potential using TMRM probe, (e) membrane integrity by measuring the extracellular LDH, (f) DNA damage by histone H2AX phosphorylation (exclusively in hepatocytes) and (g) imaging of neurite outgrowth (exclusively in neurons).

The assays were performed 24 hours after cell collection and isolation. For apoptosis, TMRM, HOECHST and WST-8 assays, cells were seeded at a density of 5000 cells/well in coated 96 well plates, incubated for 24 hours with the compound and then stained during 60 minutes with the following fluorescent probes: 5  $\mu$ M CellEvent Caspase 3/7 Green Detection Reagent (Invitrogen) for measuring caspase 3/7 activation, 50  $\mu$ M tetramethyl rhodamine methyl ester (TMRM probe, Anaspec Inc.) for measuring mitochondrial depolarization related to transient cytosolic  $\text{Ca}^{2+}$  signals, 5  $\mu\text{g/mL}$  of HOECHST (Sigma-Aldrich) for nuclei detection and 10  $\mu\text{L/well}$  of WST-8 reagent (Sigma-Aldrich) to detect viable cells. After 60 minutes, absorbance at 450 nm was measured in order to analyze cell viability (WST-8). After that, cells were washed three times and analyzed using the automatic fluorescence microscope BD Pathway 855. Pictures were taken using the a 20X objective and 488nm/515nm ex/em filters for CellEvent Caspase 3/7 reagent, 555nm/645nm ex/em filters for TMRM and 380nm/460nm ex/em filters for HOECHST. Data were analyzed with AttoVision (Becton Dickinson). The LDH assay was performed according to the manufacturer's protocol, Cytotoxicity Detection Kit LDH (Roche). LDH release was measured in mU/L in culture media obtained from cells subjected to treatments for 24 hours. The release of LDH from cells with damaged plasma membrane into the culture medium is assayed by incubating the clarified culture medium with sodium pyruvate in the presence of NADH. Pyruvic acid is catalyzed into lactic acid by free LDH along with a simultaneous oxidation of NADH to  $\text{NAD}^+$ . The rate of oxidation of NADH to  $\text{NAD}^+$  was measured at 490nm using Synergy 2 from BioTek.

For the neurite outgrowth assay, samples were washed with PBS and fixed using methanol for 10 minutes at  $-20^{\circ}\text{C}$ . After fixation, the samples were washed three times with PBS and permeabilized with PBS-Triton 0.3% for 10 minutes. The samples were washed three times and then blocked with BSA 0.5% in PBS for 30 minutes. Anti-tubulin III antibody (Sigma-Aldrich) was added at 1/1000 dilution in blocking solution and incubated for 60 minutes at room temperature. After three washing steps, a secondary antibody Alexa 488 was added at 1/100 and incubated for 60 minutes. After washing three times with PBS, pictures were taken using the BD Pathway 855 automated fluorescent microscope at 488nm/515nm ex/em. Neurite average length was calculated using the neurite outgrowth module of AttoVision software (Becton Dickinson).

DNA damage was evaluated by washing cells with PBS and fixation using paraformaldehyde 3% in PBS for 15 minutes. After fixation, the samples were washed three times with PBS and permeabilized with PBS-Triton 0.3% for 10 minutes. After washing, the samples were blocked with PBS-BSA 0.5% for 30 minutes. H2AX antibody (Abcam) was added at 1/400 in PBS-BSA 2.5% and incubated for 60 minutes at room temperature. After

three washing steps, the secondary antibody Alexa 488 was added 1/100 and incubated for 60 minutes. After washing three times with PBS, pictures were taken using the BD Pathway 855 automated fluorescent microscope at 488nm/515nm ex/em filters. To determine the DNA damage, intensity in the nuclei was analyzed using AttoVision software (Becton Dickinson).

Nimesulide (400  $\mu$ M) was included as a positive toxicity control, and the vehicle as a negative control. The relative percentage of deviation from the negative control was quantified and assigned with a number from 0 to 5 according to the following criteria: 0 (0-20% deviation), 1 (20-40%), 2 (40-60%), 3 (60-100%), 4 (100-1000%), or 5 (>1000% deviation). The sum of these values was further ranked to create a combined injury criteria that varied from no injury (0), low injury (1 to <5), moderate injury ( $\geq$ 5-to <12) to high injury ( $\geq$  12).

## 2.9. *In vivo* activity

To evaluate BNIPSpd efficacy *in vivo*, five to six weeks old female BALB/c mice were obtained from Charles River and infected with *T. cruzi* trypomastigotes expressing luciferase. Parasites were collected from the supernatants of a monolayer of Vero infected cells, washed and resuspended in PBS-glucose 0.2%, and injected intraperitoneally in mice ( $1 \times 10^4$  per mouse). After 7 days of infection, mice were treated with drugs for 4 consecutive days by oral route (benznidazole in 20% Kolliphor) or intravenous route (BNIPSpd in 10% DMSO) at a dose of 100 and 5 mg/kg/day, respectively. Infection and treatments efficiency were evaluated following subcutaneous injection of 2.1 mg of luciferin and through whole animal live imaging using an IVIS Lumina LT (Perkin Elmer). Images of the animals were analyzed by LivingImage (Xenogen).

## 2.10. Pharmacokinetics

The pharmacokinetic profile of BNIPSpd was determined following intravenous injection of 5mg/kg in BALB/c mice. The blood was collected from the tail veins after 0 min, 5 min, 15 min, 30 min, 45 min, 1 h, 3 h, 24 h, 48 h and 72 h of drug administration. Blood concentrations of the drug were analyzed by UHPLC-MS/MS ESI+.

### 3. Results

#### 3.1. TcSir2rp1 is a canonical sirtuin with NAD<sup>+</sup>-dependent deacetylase activity

The sequence of the putative Sir2rp1 gene from the reference, sequenced CL Brener strain [54] was retrieved from the trypanosomes genome database (accession number: XP\_818420.1). Because of the hybrid nature of the strain, two sequences are currently annotated. We cloned the Esmeraldo-like protein. Globally, there is a 3% difference to the non-Esmeraldo like protein at the amino acid level due to two additional aminoacids in the C-terminal. We PCR amplified the gene with primers designed taking the Esmeraldo-like sequence as a template. Sequencing of the cloned sequenced inserted into pGEM T-easy vector, had a 100% match with the Esmeraldo-like gene.

The gene was then cloned into pET-28a in order to yield a C-terminal hexa-histidine tailed protein. *E. coli* BL21 (DE3) transformed with the resulting plasmid produced soluble protein in a range of conditions, with the highest quantity being produced when the bacteria were induced overnight at 18°C with 0.5 mM of IPTG. Recombinant TcSir2rp1 was separated from other bacterial proteins by purification with an affinity chromatography and imidazole was removed by buffer exchanging to PBS. The purified protein was run on SDS-PAGE gel stained with Coomassie blue and found to be of the appropriate size (approximately 40 kDa), and with only residual impurities observable when 10 µg of the protein were loaded (Fig. 2A). Additionally, protein Western blotting with an anti-HisTag antibody also revealed a single band with the expected molecular weight (Fig. 2A).

To detect deacetylase activity, we used a commercial kit, SIRT1/Sir2 Deacetylase Fluorometric Assay Kit (CycLex, Japan). The reaction principle involves the deacetylation of a synthetic peptide substrate (Ac-peptide) that in the deacetylated form becomes the substrate for a lysylendopeptidase that releases a quencher group from the molecule, allowing the fluorophore to emit fluorescence. Deacetylase activity was detected with 0.5 µg of protein in the presence of 200 µM of NAD<sup>+</sup> and 10 µM of acetylated peptide substrate (Fig. 2B). However, when NAD<sup>+</sup> was excluded from the reaction, there was no increase in fluorescence, confirming TcSir2rp1 as a NAD<sup>+</sup>-dependent deacetylase (Fig. 2B). Furthermore, when incubated with the histone deacetylase class I and II inhibitor trichostatin A (TSA) at 1 µM, TcSir2rp1 still displayed 77±8 % of activity (Fig. 2B). TSA is a poor inhibitor of sirtuins (class III deacetylases), confirming the classification of the protein as a canonical Sir2.

The kinetics of the deacetylase activity were determined by performing the reactions with one of the reactants fixed in an excess concentration, while the concentration of the other reactant was varied, in order to have steady state conditions. When NAD<sup>+</sup> was fixed

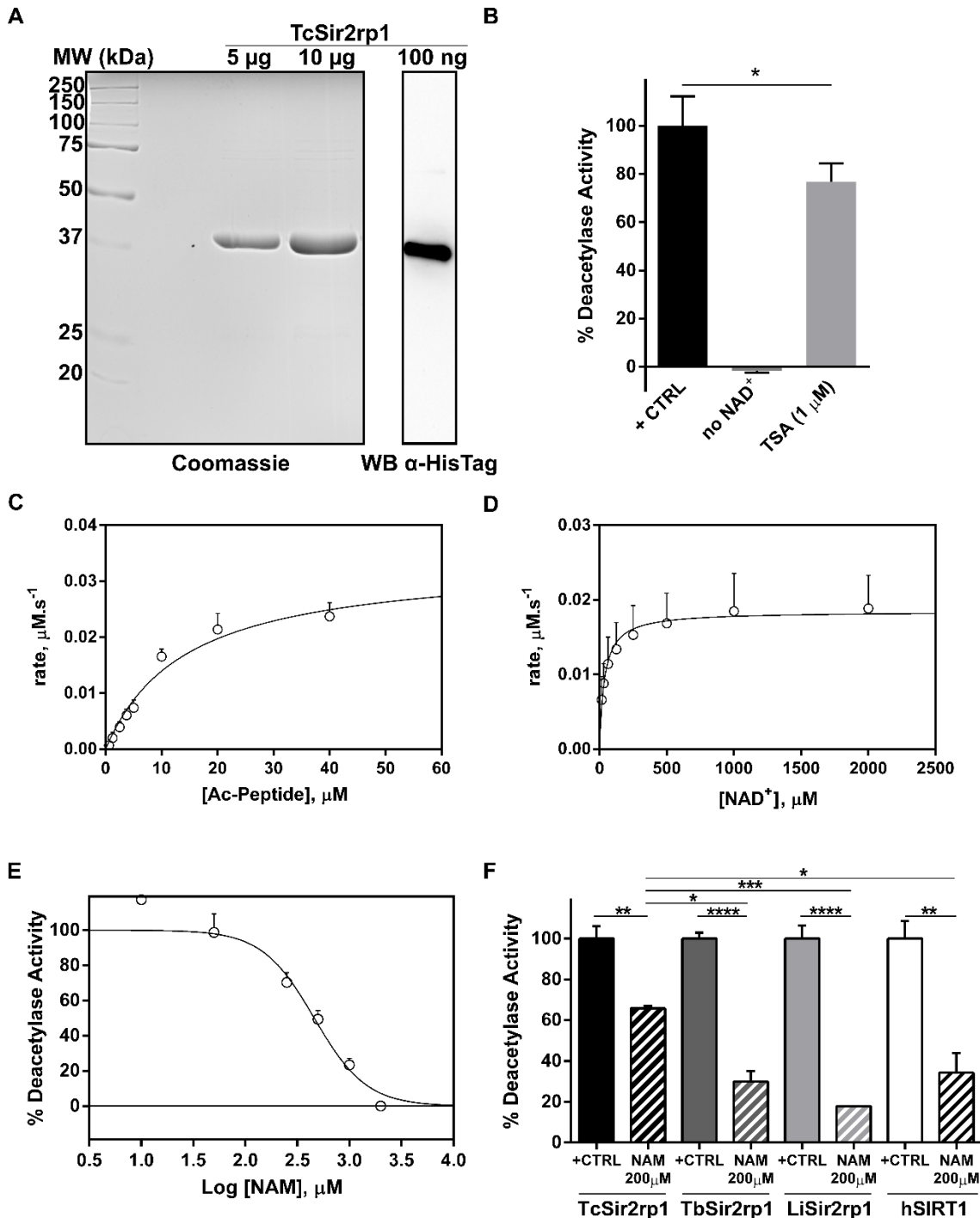
at 2000  $\mu\text{M}$ , acetylated peptide was varied from 0.63 to 40  $\mu\text{M}$ , and when the latter was kept at 40  $\mu\text{M}$ ,  $\text{NAD}^+$  was varied from 15.63 to 2000  $\mu\text{M}$ . The initial velocities represented as  $\mu\text{M}\cdot\text{s}^{-1}$  were then plotted for each condition (Fig. 2C and D). The data was analyzed by curve fitting the Michaelis-Mentel model available in GraphPad Prism Software 6.0.  $K_m$ ,  $v_{\text{max}}$ ,  $k_{\text{cat}}$  and  $k_{\text{cat}}/K_m$  values calculated are shown in Table 1. The  $\text{NAD}^+$  kinetic data obtained for this enzyme is highly similar to the data obtained for *T. brucei* Sir2rp1 [49]. Furthermore, comparing with other characterized sirtuins like yeast Sir2 and the human SIRT2, TcSir2rp1 also displays a high deacetylation activity as demonstrated by the catalytic efficiency ( $K_m/k_{\text{cat}}$ ) constants calculated for the homologue enzymes [193, 200][55, 56]. Catalytic efficiencies are the most relevant constant in physiological conditions since they define the rate of the reaction when substrate concentrations are not at saturating levels, like what happens for most cellular enzymatic reaction [56].

**Table 1**  
Kinetic parameters determined for TcSir2rp1

	Ac-Peptide	$\text{NAD}^+$
$K_m$ ( $\mu\text{M}$ )	$14.23 \pm 0.61$	$38.32 \pm 9.64$
$v_{\text{max}}$ ( $\mu\text{M}\cdot\text{s}^{-1}$ )	$0.034 \pm 0.003$	$0.018 \pm 0.004$
$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$0.136 \pm 0.013$	$0.075 \pm 0.018$
$k_{\text{cat}}/K_m$ ( $\text{mM}^{-1}\cdot\text{s}^{-1}$ )	9.585	1.956

Taking into account our results, we established a screening assay where reactants were fixed at 10  $\mu\text{M}$  (Ac-peptide) and 200  $\mu\text{M}$  ( $\text{NAD}^+$ ).

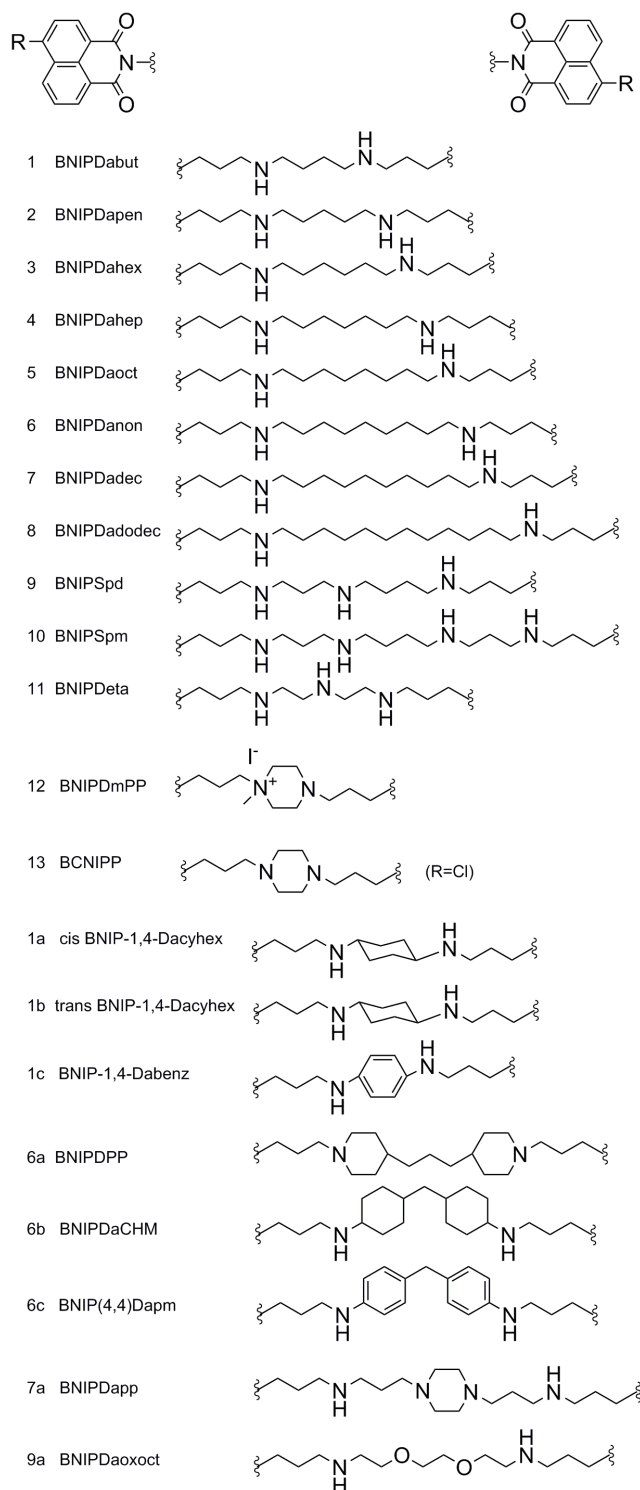
Nicotinamide was tested in order to evaluate if small molecules could inhibit the enzymatic activity described. Nicotinamide is a classic non-competitive inhibitor of sirtuins that has been used to characterize several enzymes [22, 57]. A dose-response curve of nicotinamide inhibition of TcSir2rp1 indicates an  $\text{IC}_{50}$  of  $456 \pm 44$   $\mu\text{M}$  (Fig. 2E). This value is significantly higher when compared to the  $\text{IC}_{50}$  determined for other sirtuins like the human SIRT1 ( $118.3 \pm 23.6$   $\mu\text{M}$ ), the *Plasmodium falciparum* Sir2A ( $51.2 \pm 3.0$   $\mu\text{M}$ ) or the LiSir2rp1 ( $39.4 \pm 5.0$   $\mu\text{M}$ ) previously described [46, 58]. When a concentration of 200  $\mu\text{M}$  of nicotinamide was tested against TbSir2rp1, LiSir2rp1 and the human SIRT1, TcSir2rp1 only had a mild inhibition of  $34 \pm 1\%$  whereas the inhibition was  $70 \pm 5\%$ ,  $82 \pm 0\%$ ,  $66 \pm 10\%$  for TbSir2rp1, LiSir2rp1 and human SIRT1, respectively (Fig. 2F). Some studies have previously reported the antiparasitic activity of nicotinamide on *T. cruzi*, but, to our best knowledge, this is the first report that proves the molecular inhibition of the parasite sirtuin by nicotinamide [45, 59].



**Figure 2.** *Trypanosoma cruzi* Sir2rp1 characterization and inhibition by nicotinamide **A**) Purity analysis of 5 and 10  $\mu$ g of TcSir2rp1 by SDS-PAGE stained with Coomassie brilliant blue (left panel). Western Blot analysis of 100 ng of the hexa-histidine tailed TcSir2rp1 recombinant protein with an anti-HisTag antibody. **B**) TcSir2rp1 deacetylase activity was measured with a fluorimetric kit in the presence or absence of  $\text{NAD}^+$  (+CTRL), and in presence of 1  $\mu\text{M}$  of trichostatin A. Bars represent mean + standard deviation. Data of 3 independent experiments. **C**) Deacetylation reactions for the determination of the kinetic constants of Ac-peptide.  $\text{NAD}^+$  was fixed at 2000  $\mu\text{M}$  while Ac-peptide was varied (0.63 to 40  $\mu\text{M}$ ). Plots of initial velocities versus [Ac-peptide] were fitted to the Michaelis-Menten equation, yielding the kinetic constants (see Table 1). **D**) Deacetylation reactions for the determination of the kinetic constants of  $\text{NAD}^+$ . Ac-peptide was fixed at 40  $\mu\text{M}$  while  $\text{NAD}^+$  was varied (15.63 to 2000  $\mu\text{M}$ ). Plots of initial velocities versus [ $\text{NAD}^+$ ] were fitted to the Michaelis-Menten equation, yielding the kinetic constants (see Table 1). Dots and error bars represent mean + standard



deviation. C-D) Data of 3 independent experiments. **E)** Dose-response curve of TcSir2rp1 inhibition by nicotinamide. Data represents the average + standard deviation of three independent experiments. **F)** Differential inhibition of TcSir2rp1, TbSir2rp1, LiSir2rp1 and hSIRT1 by a dose of 200  $\mu$ M of nicotinamide. Bars represent mean + standard deviation. Data of 2 independent experiments. Differences between the experimental groups were considered significant as follows: \*  $p < 0.05$ , \*\*  $p < 0.005$ , \*\*\*  $p < 0.001$  and \*\*\*\*  $p < 0.0001$ .

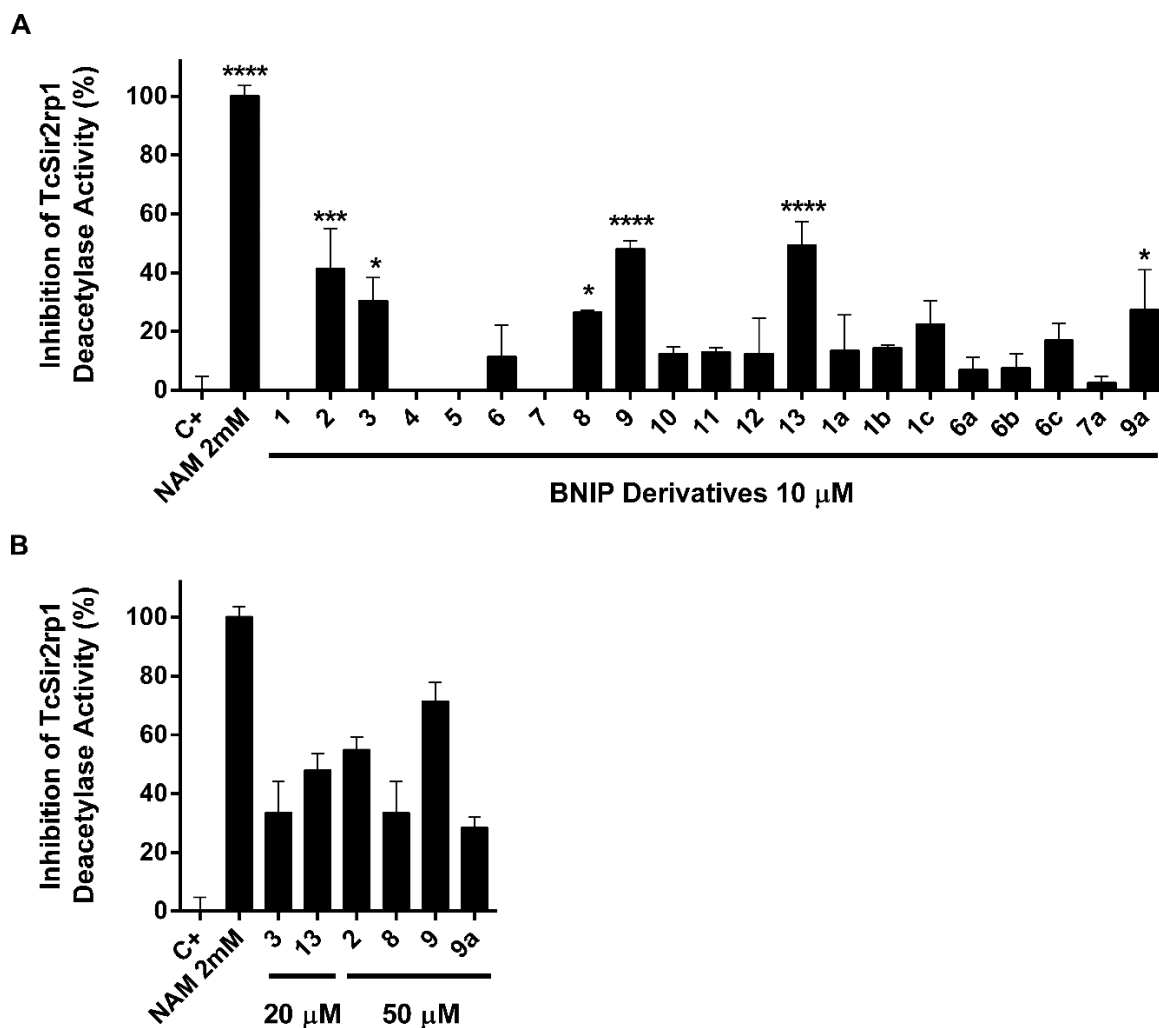


### 3.2. Inhibitory activity of BNIP derivatives on TcSir2rp1

We next evaluated the enzymatic inhibition of TcSir2rp1 by a class of experimental compounds previously characterized as inhibitors of the orthologue enzyme of *L. infantum* [46] as well as the newly synthesized derivatives. BNIP derivatives are constituted by two naphthalimidopropyl groups separated by a linker that varies in length and functional groups, and have been characterized as  $\text{NAD}^+$ -competitive inhibitors of LiSir2rp1 [60]. In this study, an additional set of compounds was synthesized with the objective of improving cellular target binding by including heteroatoms in the alkyl chain connecting the two naphthalimidopropyl groups, as well as by introducing cyclic structures in the linker (Figure 3). The results are summarized in Figure 4 A and indicate the percentage of inhibition of the  $\text{NAD}^+$ -dependent deacetylase activity for a single dose concentration of 10  $\mu$ M. Of all the compounds tested, compounds 2, 3, 8, 9, 13 and 9a had a statistically significant inhibition of the enzyme (1-way ANOVA). In order to

**Figure 3.** Chemical structures of the bisnaphthalimidopropyl derivatives used in the present study.

verify a concentration dependent effect on enzymatic inhibition, compounds 2, 8, 9 and 9a were additionally tested at 50  $\mu$ M, whereas compound 3 and 13 were tested at 20  $\mu$ M due to low solubility. Inhibition percentages for this higher concentration were  $55\pm4\%$ ,  $34\pm11\%$ ,  $31\pm9\%$ ,  $72\pm6\%$ ,  $48\pm6$  and  $29\pm4\%$  for compounds 2, 3, 8, 9, 13 and 9a, respectively (Fig4B). Only compound 9 demonstrated a statistically significant dose-dependent increase in inhibition (1-way ANOVA, p value  $<0.05$ ). In fact, compound 9 was one of the most active compounds against TcSir2rp1, with a 10  $\mu$ M dose inhibiting almost 50% of the NAD<sup>+</sup>-dependent deacetylase activity, a concentration 45 times lower than the necessary for nicotinamide to achieve the same level of inhibition. Compound 9 is a derivative of compound 5 by substitution of one of the carbons in the linker chain by a nitrogen atom. Since compound 5 was not active against the enzyme, it is possible that the substitution of the nitrogen allows the establishment of additional molecular interactions with the enzyme and/or modifies the rigidity of the linker chain resulting in higher inhibition. Compound 9a is also a derivative from compound 5 with eight atoms in the linker chain separating the two naphthalimidopropyl groups, but with two oxygen atoms in substitution of two carbons. This modification also seems to increase the inhibitory activity against TcSir2rp1 ( $28\pm13\%$ ) but it is not as marked as for compound 9. Although a clear correlation between the length of the linking chain and enzymatic inhibition was established for the *L. infantum* orthologue [46], the same was not verified with TcSir2rp1. Compounds with 5 and 6 carbons in the linker chain also displayed some inhibitory activity against the enzyme (compound 2,  $42\pm13\%$  and 3,  $31\pm8\%$ , respectively), but when tested at a higher concentration, the increase was not statistically significant. Compound 3 was only tested at 20  $\mu$ M due to poor solubility. In general, the most active inhibitors of TcSir2rp1 were also selective towards the *T. cruzi* enzyme, as demonstrated by inhibition of a human sirtuin homologue, hSIRT1. In addition to hSIRT1 inhibition previously described for compounds 1-11 [46], compounds 12-9a were also tested at 10  $\mu$ M against this enzyme (Table S1). Compound 6b was the most active against the human homologue enzyme ( $44\pm8\%$  of inhibition), but was a weak inhibitor of TcSir2rp1 ( $8\pm5\%$ ) at the same concentration of 10  $\mu$ M. Compound 13 also had a reduced activity against hSIRT1 ( $15\pm4\%$ ), but was a much stronger inhibitor of the *T. cruzi* enzyme, with an inhibition at least 3-fold higher ( $50\pm8\%$ ).

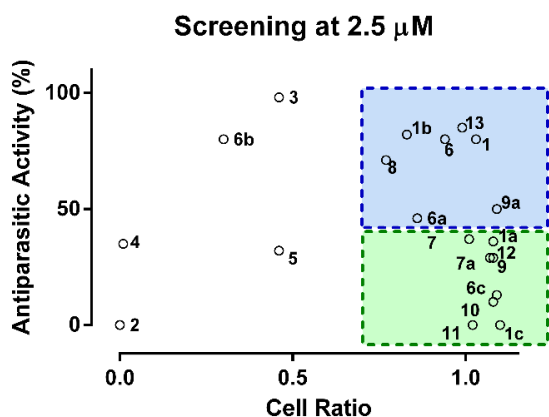
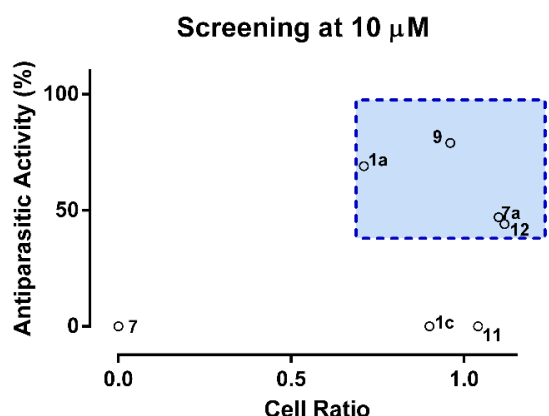
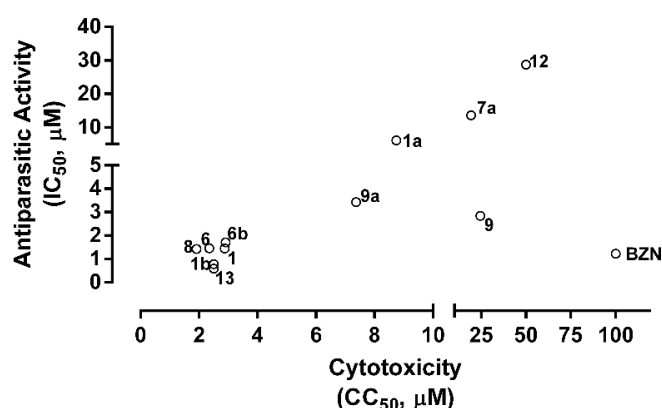


**Figure 4.** Enzymatic inhibition of TcSir2rp1 by BNIP derivatives. **A-B)** The percentage of inhibition of the NAD<sup>+</sup>-dependent deacetylase activity of TcSir2rp1 by BNIP derivatives is represented in the y axis. A positive (no drug) and negative (NAM, nicotinamide at 2 mM) controls are represented. Bars represent the average + standard deviation of at least two independent experiments. Differences between the experimental groups were considered significant as follows: \*  $p < 0.05$ , \*\*\*  $p < 0.001$  and \*\*\*\*  $p < 0.0001$ .

### 3.3. BNIPs are potent *in vitro* inhibitors of *Trypanosoma cruzi* intracellular amastigotes

Once demonstrated that the deacetylase enzymatic activity is inhibited by some BNIP derivatives, the compounds were also tested against whole parasites with an *in vitro* assay using HCS and the intracellular form of amastigotes (Fig. S1). In this assay, the compounds are incubated for 72 hours with cells previously infected with wt trypomastigotes for 24 hours. The readout is done by comparing the average number of amastigotes per cell that developed by the end of the incubation period. The quality of the assay was statistically evaluated by the calculation of the z-factor [61], a calculation that represents the reliability of the assay in distinguishing positive controls confidently from negative controls.

It varies from 0 to 1, with values higher than 0.5 considered being acceptable for drug screening. The z-factor obtained was of 0.68, validating the use of the assay (Fig. S1B). The reference drug benznidazole was assayed for quality control and showed an  $IC_{50}$  of  $1.23 \pm 0.30 \mu M$ , in accordance with previous studies [62].

**A****B****C**

**Figure 5.** *In vitro* activity of BNIP derivatives. **A)** Primary screening of BNIP derivatives by an *in vitro* assay for intracellular *T. cruzi* amastigotes at a single dose of 2.5  $\mu M$ . Values for both anti-parasitic activity and cell ratio are represented in the dot plot graph. The blue square represents the zone of selection for active, low toxicity hits to be evaluated by dose-response curve analysis. The green square represents the zone of selection for compounds to be tested at a higher dose. **B)** Primary screening of the compounds selected for testing at a higher dose of 10  $\mu M$ . Values for both anti-parasitic activity and cell ratio are represented in the dot plot graph. The blue square represents the zone of selection for active, low toxicity hits to be evaluated by dose-response curve analysis. **C)** Representation of the selectivity indexes of the compounds analyzed by dose-response curve analysis, with the determined antiparasitic activity ( $IC_{50}$ ) in the y axis, and the cell ratio ( $CC_{50}$ ) in the x axis. Dots represent the average of three independent experiments.

In order to prioritize compounds, all the BNIP derivatives were firstly screened at a single dose of 2.5  $\mu M$  (Fig 5A). The compounds that presented high anti-parasitic activity (>40%) and low toxicity (high cell ratio, defined as [average number of cells for the compound / average number of cell for the DMSO 0,5% control], >70%), represented in a blue box, were selected for dose-response curve analysis. A set of compounds that was

not toxic at 2.5  $\mu M$  (green box) was further tested at 10  $\mu M$ . Again, the compounds that had high anti-parasitic activity and low toxicity were selected for dose-response analysis (blue box, Fig. 5B). Compounds 10 and 6c were not tested at 10  $\mu M$  due to poor solubility.

Compounds 1, 6, 8, 9, 12, 13, 1a, 1b, 6a, 7a and 9a (Fig 5C and Table 2) were assayed by dose-response curves in the same conditions of the primary screening. Compound 9, the strongest inhibitor of TcSir2rp1 was also active against *T. cruzi* amastigotes, presenting an  $IC_{50}$  of  $2.84 \pm 0.30 \mu M$ , in the range of the reference drug benznidazole ( $1.23 \pm 0.30 \mu M$ ). In addition, it was the least toxic of the compounds analyzed by dose-response curve, with a  $CC_{50}$  of  $24.47 \pm 0.45$ , resulting in a selectivity of 8.8 units. Compound 9a, an analogue of 9 with two oxygen atoms in the linking chain instead of one nitrogen atom, was also active and presented a similar  $IC_{50}$  of  $3.43 \pm 0.57 \mu M$ . However, it was less selective than compound 9 (2.1 units). Compound 13, also an inhibitor of TcSir2rp1 ( $50 \pm 8\%$ ), was the most active compound against *T. cruzi* amastigotes, with an  $IC_{50}$  of  $0.59 \pm 0.23 \mu M$ . It was not possible to calculate the  $CC_{50}$  since the highest concentration tested allowed by the solubility of the compound ( $2.5 \mu M$ ) did not reduce the cell ratio below 50%, making the selectivity of at least 4.2. The second most potent hit, compound 1b, is a derivative of compound 1 where a cyclohexane cycle was introduced as a spacer between the naphthalimidopropyl groups. The synthesis of this compound was performed with two isomers with a significant difference in their antiparasitic activity, where the trans isomer (compound 1b) presents an  $IC_{50}$  of  $0.78 \pm 0.12 \mu M$  and the cis isomer (compound 1a) an  $IC_{50}$  of  $6.09 \pm 0.14 \mu M$ . Such difference suggests that particular stereoisomeric configurations improve antiparasitic activity. Activity of nicotinamide in the amastigote assay was also assessed, but there was no activity detected up to a concentration of  $2000 \mu M$  (data not shown).

**Table 2**  
Antiparasitic activity, cytotoxicity and selectivity index of BNIP derivatives selected for dose-response curve analysis.

Compound	Anti- <i>T. cruzi</i> activity Amastigotes $\mu M$ ( $IC_{50} \pm SD$ )	C2C12 cytotoxicity $\mu M$ ( $CC_{50} \pm SD$ )	Selectivity Index*
1 BNIPDabut	$1.71 \pm 0.23$	$2.91 \pm 0.94$	1.7
6 BNIPDanon	$1.45 \pm 0.37$	$2.35 \pm 0.77$	1.7
8 BNIPDadodec	$1.43 \pm 0.10$	$1.91 \pm 0.06$	1.3
9 BNIPSpd	$2.84 \pm 0.18$	$24.47 \pm 0.45$	8.8
12 BNIPDmPP	$28.74 \pm 4.09$	> 50	> 1.7
13 BCNIPP	$0.59 \pm 0.23$	> 2.50	> 4.2
1a cis BNIP-1,4-Dacyhex	$6.09 \pm 0.14$	$8.74 \pm 1.14$	1.4
1b trans BNIP-1,4-Dacyhex	$0.78 \pm 0.12$	> 2.50	> 3.2
6a BNIPDPP	$1.45 \pm 0.30$	$2.88 \pm 0.12$	2.0
7a BNIPDapp	$13.58 \pm 2.90$	$19.30 \pm 5.64$	1.4
9a BNIPDaooct	$3.43 \pm 0.57$	$7.37 \pm 0.65$	2.1
Benznidazole	$1.23 \pm 0.30$	> 100	> 81.0

\*Selectivity Index =  $CC_{50}$  cell line/ $IC_{50}$  *T. cruzi*

### 3.4. BNIPSpd failed in a preclinical mice model for Chagas disease

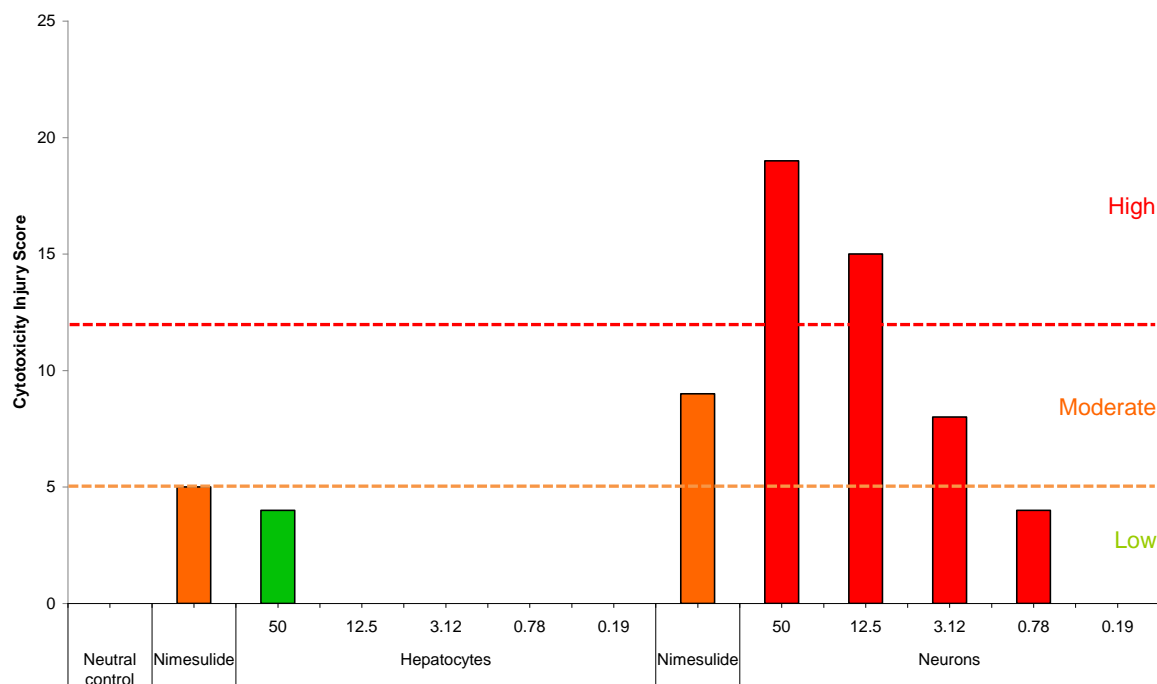
Compound 9, BNIPSpd presented high enzymatic inhibition, low IC<sub>50</sub> against the parasite and also higher selectivity, and based on these parameters was chosen for follow up characterization. The compound toxicity was further evaluated against a panel of primary cells to determine tissue specific toxic effects. The results of cytotoxicity against neurons, hepatocytes and MDCK cells, and the cardiotoxicity determination by hERG predictor method are summarized in Table 3. As shown, BNIPSpd presents hepatotoxicity and neurotoxicity values very similar to its efficacy concentration (SI 1.1 and 1.4 respectively). On the other hand, it seems not to be nephrotoxic (SI 35.2) nor cardiotoxic (hERG channel 99.6% functional). The toxicity values are higher than the corresponding ones for the C2C12 cells (Table 2 SI=8.8) but this is not surprising given the fact that the primary cells are usually more sensitive. Furthermore, the toxic effects at the cellular level were evaluated and quantified by a set of *in vitro* assays that include mitochondrial dysfunction, membrane integrity, DNA damage, apoptosis and neurite outgrowth on hepatocytes and neurons (Figure 6). This analysis could not be performed in the MDCK cell line due to the high amounts of DMSO to test the required BNIPSpd concentration and that were toxic for the cells.

**Table 3**  
Cytotoxicity and selectivity of BNIPSpd

Cell type	Cytotoxicity	Selectivity Index*
Hepatotoxicity (Hepatocytes)	≥ 3.1 μM	1.1
Neurotoxicity (Neurons)	≥ 3.9 μM	1.4
Nephotoxicity (MDCK cells)	≥ 100 μM	35.2
Cardiotoxicity (hERG predictor)	0.36 ± 0.51 %	-

\*Selectivity Index = CC<sub>50</sub> cell line / IC<sub>50</sub> *T. cruzi*

Nimesulide, an approved and widely used anti-inflammatory drug was included at a concentration of 400 μM as a control of toxicity [63-65]. The results are presented in Fig. 6. In summary, the BNIPSpd showed a low injury score for hepatocytes and moderate injury score for neurons. These values correspond to the range within the efficacy concentration for this compound (2.84 μM). Despite showing a mild injury score for neurons, it should be kept in mind that further analysis need to be done in order to determine whether the compound is able to cross or not the blood-brain barrier. Indeed, a compound could be very neurotoxic but never been able to reach the neurons.



**Figure 6.** *In vitro* hepatocytes and neurons injury scores for BNIPSpd by HCS. The score was calculated as the sum of individual scores obtained from a panel of *in vitro* cytotoxicity assays that include; mitochondrial dysfunction measured by TMRM probe dynamics in cells, membrane integrity assayed by lactate dehydrogenase quantification, DNA damage by imaging with H2AX antibody and apoptosis by caspase 3/7 activation or neurite outgrowth as imaged with an anti-tubulin III antibody. Nimesulide (400  $\mu$ M), an approved drug with a mild toxicological profile, was included as a toxicity control.

Finally, BNIPSpd *in vivo* activity was characterized with a murine model of *T. cruzi* infection and using bioluminescent imaging. A bioluminescent *T. cruzi* Y strain was obtained like previously described [47] and its *in vitro* limit of detection in the bioimaging equipment IVIS Lumina LT was determined to be of  $10^4$  parasites (t-test, p-value < 0.05) (Fig. S2A). Different numbers of Luc<sup>+</sup> trypomastigotes were tested in order to access which condition yielded the best readout (Fig. S2B). When injected intraperitoneally with  $10^4$  parasites, the mice developed an infection at first mostly located in the site of infection (day 7) but that later spreads in the whole body (Fig. S2B). Similar observations were made for  $10^5$  and  $10^6$  inocula (data not shown), but  $10^4$  parasites yielded a higher and most reproducible increase in the bioluminescent signal as measured by the whole body bioluminescent signal in average radiance.

A regimen of 5 mg/kg/day of BNIPSpd was administered intravenously for 4 consecutive days, while a positive control with a dose of 100 mg/kg/day of benznidazole *per os* and the respective vehicle controls were also performed (Fig. S3A). Unfortunately, BNIPSpd did not exhibit any *in vivo* activity, since bioimaging analysis demonstrates the infection progressed like in the animals treated with the vehicles that on average increased

the luminescence signal by 40-fold. By comparison, animals treated with benznidazole at 100 mg/kg/day displayed, on average, a 700-fold reduction in the bioluminescence signal compared to vehicle treated mice (Fig. S3A).

To investigate whether the lack of antiparasitic activity *in vivo* would be due to BNIPSpd pharmacokinetics profile we proceed to compound quantification in the blood over the time. Analysis of the snapshot pharmacokinetics profile (Fig. S3B) may explain the lack of *in vivo* activity of BNIPSpd. For the first 30 minutes after intravenous injection of the compound, we verified that it never reaches a concentration equivalent to the *in vitro* IC<sub>50</sub> for *T. cruzi* amastigotes. The highest concentration detected in the plasma is 1.70 µM at the 5 minutes time-point, and the compound is no longer detected after the 3 h time-point and up to 72 h post administration (lower limit of quantification is 8.1 nM). This pharmacokinetic profile indicates that a concentration of drug able to reach and clear parasitized tissues is not achieved in the mice.

#### 4. Discussion

This work sought to explore the potential of inhibitors of TcSir2rp1, as possible bioactive compounds against the infection caused by this parasite. Sirtuins have long been proposed to be interesting targets to treat parasitic diseases [66, 67], but only recently have these proteins been characterized in *T. cruzi* [44, 45]. Even though the localization, expression and some of the important functions of *T. cruzi* sirtuins have been described, no biochemical characterization has been made to date.

Our results demonstrate that the annotated coding sequence of TcSir2rp1 encodes a canonical sirtuin that does not display deacetylation activity in the absence of NAD<sup>+</sup> and is mostly insensitive to TSA. TSA is usually included in sirtuin deacetylation activity assays to exclude the contribution of other histone deacetylases present in a study sample. Although in this study 1 µM of TSA resulted in a slightly higher inhibition when compared with other orthologue enzymes (see [22] and the hSIRT1 deacetylation activity described in the manufacturer's manual), the lack of activity in the absence of NAD<sup>+</sup> excludes the possibility of a non-sirtuin source of activity.

The kinetic data obtained for TcSir2rp1 is highly similar to the values described for TbSir2rp1, despite overall sequence similarity being only 61%. However, when the core sirtuin Pfam domain of both proteins are aligned, identity level increases to 73%. A close analysis to the amino acids adjacent to the essential histidine (H142) at the catalytic site, demonstrates a 100% similarity of the four amino acids upstream and four downstream that likely participate and/or help shape the catalytic site and thus may explain the similarity



observed. Nonetheless, it should be noted that distant amino acids in terms of primary structure may have an interference on enzymatic activity, as demonstrated in the case of PfSir2 in which the C- and N-terminal removal led to modifications in the enzyme kinetic constants and sensitivity to nicotinamide [58].

An essential step in the evaluation of a drug target is the proof of druggability. Even if a gene product is deemed essential for a given organism, if the activity or function mediated by this protein is not amenable to modulation by small molecule inhibitors, then it is not considered to be a good drug target. To this end, it was sought to modulate TcSir2rp1 with a classic inhibitor of this protein family, nicotinamide [68]. The enzymatic activity described was indeed inhibited by nicotinamide, although in concentrations higher than those previously reported for other members of the family. In particular, the IC<sub>50</sub> for LiSir2rp1 and hSIRT1 is 11 and 4-fold lower, respectively, than the one here reported for TcSir2rp1 [46]. Another observation that supports the resistance to nicotinamide is the fact that even when 2 mM of NAD<sup>+</sup> is used in reactions for the determination of the kinetic constants for NAD<sup>+</sup>, there is no decrease in enzymatic activity like previously reported for other enzymes [22]. Such inhibition would be expected since nicotinamide is an endogenous product of the deacetylation reaction. Nicotinamide binds to a distinct C pocket that appears to be conserved among sirtuins that have been structurally elucidated by co-crystallography [57]. The study of the presence and conservation of this pocket in TcSir2rp1 may shed some light on the resistance for nicotinamide. Although nicotinamide has some anti-parasitic activity against *Plasmodium* [69], *Leishmania* [70], *T. brucei* [71] and also *T. cruzi* [59], no confirmation of a sirtuin-mediated mechanism has been clearly established to date. In this study, no anti-parasitic activity was detected for nicotinamide in concentrations up to 2 mM.

The search for additional inhibitors led to the identification of some BNIP derivatives as stronger inhibitors of TcSir2rp1. Also inhibitors of LiSir2rp1, this series of compounds were, with few exceptions active at 10 µM, with some compounds achieving 50% of inhibition. No correlation has been established between the most active compounds for *L. infantum* and *T. cruzi* enzymes, indicating that both enzymes might be differentially targeted by some of the derivatives belonging to this class of compounds [46]. In contrast to LiSir2rp1 inhibition, no structure-activity relationship regarding the length of the alkyl chain linking the two naphthalimidopropyl groups was observed for TcSir2rp1 [60]. Although a linker of eight carbon atoms had no activity at 10 µM (compound 5), when some of the carbon atoms were substituted by heteroatoms like nitrogen (compound 9) and oxygen (compound 9a), the activity towards TcSir2rp1 increased, suggesting that the rigidity and/or the ability to establish additional molecular interactions in this part of the molecule may increase inhibitory activity.

More importantly, some enzymatic inhibitors also demonstrated a strong anti-parasitic activity against *T. cruzi* amastigotes, the clinically relevant form responsible for infection persistence in humans. Differences observed between the enzymatic inhibition and the activity in the parasite may be due to distinct interactions between BNIPs and TcSir2rp1, or, possibly, by the contribution of a multi-target mechanism. The majority of the compounds demonstrated low selectivity for the parasite and were also toxic for host cells as indicated by the selectivity indexes calculated. A possible explanation is the characteristic DNA intercalation of BNIP derivatives that were originally developed as anti-cancer agents [72, 73]. While this might explain some of the toxic effects observed for host cells, it should not be excluded that DNA intercalation may be contributing to the anti-parasitic activity reported, especially since trypanosomes are susceptible to intercalating agents [74]. Analysis of TcSir2rp1 by Wregex and cNLS Mapper, bio-computational tools that identify nuclear export signals (NES) and nuclear localization signals (NLS), respectively, indicate the presence of non-canonical NES/NLS in the sequence of this sirtuin [75, 76]. Whether TcSir2rp1 does shuttle to nucleus during specific times of *T. cruzi* life cycle, possibly to perform DNA damage repair like the orthologue from *T. brucei*, remains to be reported. In fact, if TcSir2rp1 also has a role in DNA repair like its *T. brucei* orthologue [42], both sirtuin inhibition and DNA intercalation may be synergistically contributing to the activity observed. Activity towards other molecular targets should also not be excluded, especially considering that *T. cruzi* has another sirtuin, TcSir2rp3, that has also been described as having important functions in different processes of the parasite life cycle like metacyclogenesis, epimastigote growth and host-cell infectivity and replication [44, 45]. Since BNIPSpd met some of the biologic criteria recommended for *T. cruzi* drug discovery follow-up, it was decided to further evaluate it as a drug lead [77].

The toxicity determination showed that although it has low SI values (around 1 for hepatocytes and neurons), the toxicity values in the  $\mu\text{M}$  range could be considered a promising starting point for further optimization in SAR rounds. Moreover, the cardiotoxicity evaluation, showed that the cardiomyocytes function seems not to be compromised. On the other hand, a more in depth analysis of the toxicity by HCS showed that there is a very limited injury to hepatocytes and neurons. The BNIPSpd showed no hepatotoxicity up to  $12.5\mu\text{M}$ . At  $50\mu\text{M}$  there is a low injury score mainly due to caspase 3/7 activation (although the viability -WST8- is also altered). In regards to neurotoxicity, as expected, the neurons start to suffer toxicity at lower concentrations; up to  $0.7\mu\text{M}$  the neurons showed no effects but the injury score starts to raise in a dose dependent manner up to  $12.5\mu\text{M}$ . At higher concentrations ( $50\mu\text{M}$ ) the injury score is maintained at high levels. The mechanism of toxicity in neurons seems to be the same than the one for hepatocytes, being the caspase

3/7 activation the main parameter, followed by membrane integrity and then at higher concentrations all the parameters tested were affected. More assays need to be performed to evaluate the capacity of the BNIPSpd to cross the blood-brain barrier. In addition, BNIPSpd showed decent aqueous solubility (up to 70%) and gastric stability (100%) (data not shown).

Assessment of *in vivo* efficacy of BNIPSpd by imaging mice infected with bioluminescent *T. cruzi* parasites revealed lack of activity in this mouse model. The results may be explained by the pharmacokinetics profile of the compound, that is always in a blood concentration below the *in vitro* IC<sub>50</sub> for amastigotes and is no longer detected 3 hours after administration, even though the drug is administered by intravenous injection, the most bioavailable route. An explanation is that most of the compounds is rapidly metabolized in the organism to an inactive form. Medicinal chemistry modifications of the compound, as well as alternative formulations may improve activity and pharmacokinetics in the host organism.

## Acknowledgments

We thank Paola Minoprio and Sophie Goyard at Institut Pasteur in Paris for providing pTREX-luc used to generate the *T. cruzi* Y Luc<sup>+</sup> strain.

## Funding

The research leading to these results has received funding from: the European Community's Seventh Framework Programme under grant agreement No.602773 (Project KINDReD) and Fundação para a Ciência e Tecnologia (FCT)/Ministério da Educação e Ciência (MEC) cofunded by FEDER, partnership agreement PT2020, through the Research Unit No.4293. L.G. was supported by the Fundação para a Ciência e Tecnologia through grant SFRH/BD/81604/2011. D.M.C. was supported by KINDReD-PR301404-BD III scholarship. J.T. is an Investigator FCT funded by National funds through FCT and co-funded through European Social Fund within the Human Potential Operating Programme. N.S. and B.P.-C. are supported by fellowships from the European Community's Seventh Framework Programme under grant agreements No. 602773 (Project KINDRED) and No. 603240-2 (Project NMTryPI), respectively. The authors thank the EPSRC UK National Mass Spectrometry Facility at Swansea University for high resolution mass spectral analysis on all the new compounds.

## Competing interests

None declared

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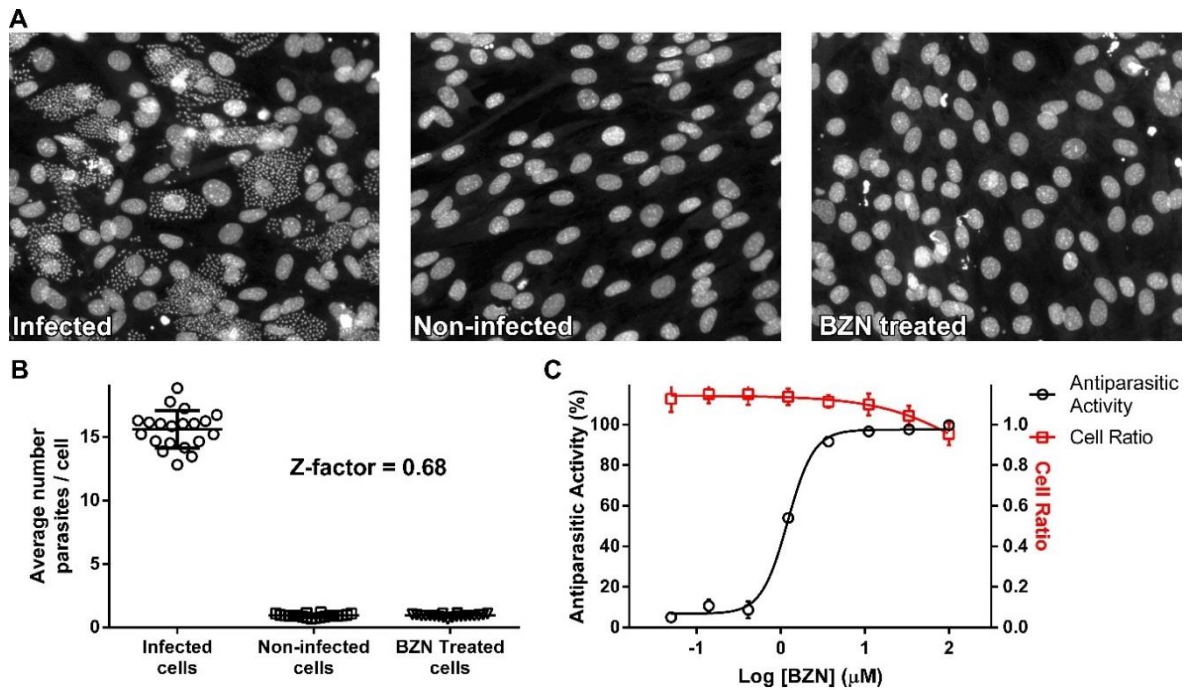
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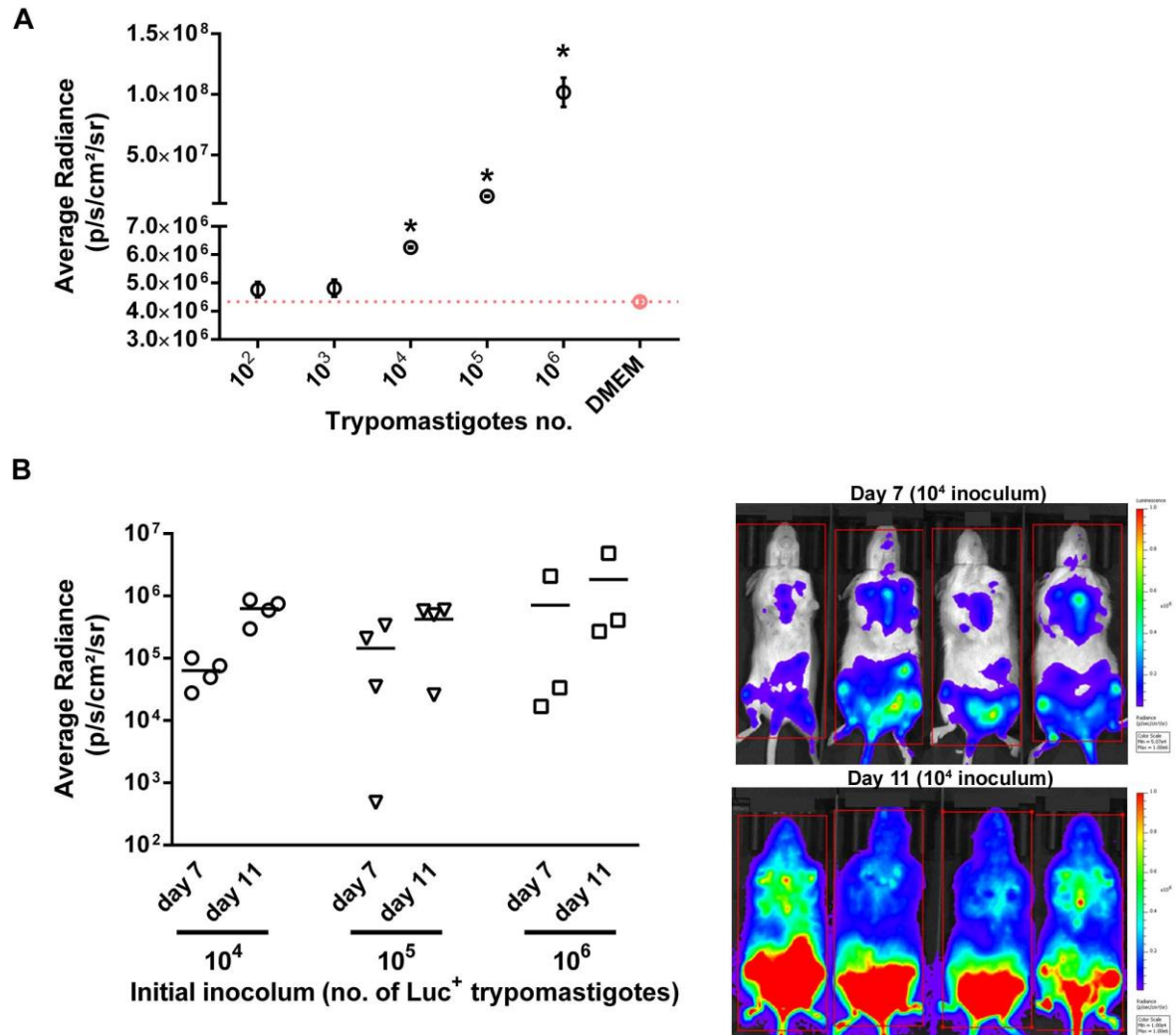


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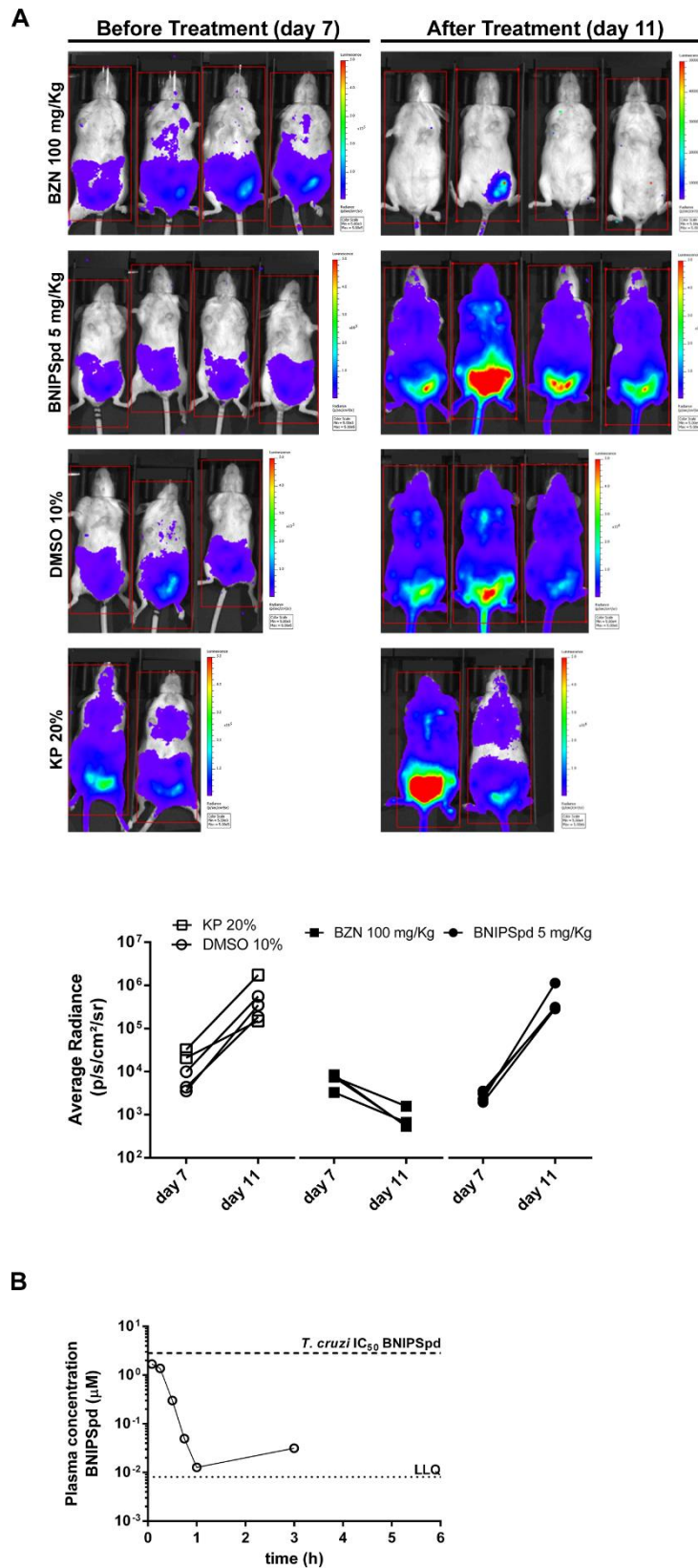
## Supplemental data



**Figure S1.** Development of the high-content screening assay against intracellular amastigotes of *Trypanosoma cruzi*. **A)** Representative images of the control conditions used in the assay: infected cells, non-infected cells and infected cells treated with 100  $\mu\text{M}$  of benznidazole. **B)** Statistical validation of the screening assay based on the readout of parasites per cell, with a Z value of 0.68. **C)** Dose-response curve against the reference drug benznidazole, demonstrating and  $\text{IC}_{50}$  in line with previously published values [62].



**Figure S2. A)** *In vitro* detection limit of Luc<sup>+</sup> trypomastigotes in a 96 well plate incubated with luciferin and imaged using the IVIS LUMINA LT. Circles and vertical lines represent the average  $\pm$  standard deviation of the average radiance of quadruplicates. Statistical analysis was performed by standard t-test relative to luciferin background with no parasites. Significance is shown in asterisks (\*,  $p$ -value  $\leq 0.05$ ). **B)** Comparison of different inocula of Luc<sup>+</sup> trypomastigotes ( $10^4$ ,  $10^5$ ,  $10^6$ ) tested in BALB/c mice infections by quantification of the signal over 5 minutes (average radiance, photons/sec/cm<sup>2</sup>/sr) and representative images of the anesthetized bio-imaged mice at the time-points before and after treatment (day 7 and 11 after infection, respectively).



**Figure S3.** *In vivo* efficacy testing of BNIPSpd. **A)** Mice were infected with  $10^4$  luc<sup>+</sup> trypomastigotes by intraperitoneal injection. Treatments (BZN, benznidazole at 100 mg/kg/day *per os*, and BNIPSpd 5 mg/kg/day by intravenous injection) were initiated 7 days after infection, as well as the respective controls (KP, koliphor 20% *per os* and DMSO 10% by intravenous injection). Imaging of the whole mice bioluminescence was done before treatment, at 7 days post-infection and after treatment, at 11

days post-infection, using an IVIS LUMINA LT and upon injection of 2.1 mg luciferin. In the lower panel, bioluminescence average radiance (photons/sec/cm<sup>2</sup>/sr) of whole mice was quantified before and after treatment. Data representative of two independent experiments. **B)** Snapshot Pharmacokinetics of BNIPSpd in BALB/c mice by quantification of BNIPSpd in the blood of mice by UHPLC-MS/MS ESI+ at different time-points after administration of a 5 mg/kg dose by intravenous injection. Data is the average + standard deviation of two independent experiments. The dashed line represents the value of IC<sub>50</sub> for BNIPSpd in the *in vitro* assay against *T. cruzi* amastigotes, and the dotted line represents the lower limit of quantification of the technique.

**Table S1**

Enzymatic inhibition of hSIRT1 by newly synthesized BNIPs

Compound	hSIRT1 inhibition at 10 $\mu$ M (% $\pm$ SD)
12	n.i.
13	15 $\pm$ 4
1a	1 $\pm$ 1
1b	3 $\pm$ 4
1c	n.i.
6a	3 $\pm$ 4
6b	44 $\pm$ 8
6c	8 $\pm$ 1
7a	n.i.
9a	3 $\pm$ 4

\*n.i. = no inhibition



#### 4.2.2.2. Activity of bisnaphthalimidopropyl derivatives against *Trypanosoma brucei*

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*Accepted for publication at Antimicrobial Agents and Chemotherapy 2016 (in press).*

##### **Objectives of the study:**

The potential activity of a set of BNIP derivatives against *T. brucei* bloodstream forms was evaluated *in vitro* and *in vivo*. A Sir2rp1-mediated mechanism of action was also evaluated.

##### **Main results:**

→ BNIP derivatives are inhibitors of *T. brucei* Sir2rp1 enzymatic activity, but it is unlikely that such inhibition is a major mechanism of action for the most active compound, BNIPDabut.

→ BNIPDabut, the most active compound, demonstrated high activity *in vitro* against bloodstream forms with IC<sub>50</sub> in the range of the reference drug pentamidine.

→ Cytotoxicity of BNIPDabut, microsomal metabolic stability and pharmacokinetic profile indicate good drug-like properties that warrant *in vivo* efficacy testing.

→ Despite high trypanocidal activity, BNIPDabut failed to cure a mouse model of acute infection.

## Conclusion:

BNIP derivatives, a class of compounds previously shown to possess antitumoral and antiparasitic activity against *L. infantum* also demonstrated to be highly active against the related trypanosomatid *T. brucei* both *in vitro* and *in vivo*. Furthermore, some of these compounds described to inhibit the *L. infantum* Sir2rp1 NAD<sup>+</sup>-dependent deacetylase enzyme also inhibited the *T. brucei* orthologue. However, the high IC<sub>50</sub> observed for BNIPDabut against the whole parasite versus the enzyme hints at other mechanisms of action taking place. Pharmacokinetic and pharmacodynamic data highlight the possibility of further exploiting this scaffold for future lead design.



AAC Accepted Manuscript Posted Online 19 January 2016  
Antimicrob. Agents Chemother. doi:10.1128/AAC.02490-15  
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# Activity of Bisnaphthalimidopropyl Derivatives Against

## *Trypanosoma brucei*

Running title: BNIP derivatives against *T. brucei*

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## 24 ABSTRACT

25 Current treatments for African trypanosomiasis are either toxic, costly, difficult to  
26 administer or prone to elicit resistance. This study evaluates the activity of  
27 bisnaphthalimidopropyl (BNIP) derivatives against *Trypanosoma brucei*. BNIPDabut, the  
28 most active compound, showed *in vitro* inhibition in the single unit nanomolar range,  
29 similar to the reference drug pentamidine, and presented low toxicity and adequate  
30 metabolic stability. Additionally, using a murine model of acute infection and live imaging,  
31 significant decrease of parasite load in BNIPDabut-treated mice was observed. However,  
32 cure was not achieved. BNIPDabut constitutes a new scaffold for antitrypanosomal drugs  
33 that deserves further consideration.

34 **MAIN TEXT**

35 African trypanosomiasis is an infectious disease caused by parasites of the species  
 36 *Trypanosoma brucei*. The parasite is transmitted by an insect vector, the tsetse fly  
 37 (*Glossina spp.*). The disease is mainly distributed in the African continent, with distinct  
 38 subspecies causing different forms of human disease: *T. brucei gambiense* infection  
 39 produces a chronic form that may last for years and was responsible for nearly 98% of the  
 40 cases in the past decade; the acute form is caused by *T. brucei rhodesiense* and usually kills  
 41 the host within weeks, accounting for the remaining 2% of the reported cases (1, 2).

42 Since vaccination remains elusive and vector control strategies are frequently  
 43 insufficient, chemotherapy is still the most efficient option to control the disease (2-5).  
 44 However, the drugs in use have many drawbacks, mostly related with cost, effectiveness,  
 45 toxicity, difficult administration and the appearance of resistance (6). Therefore, the  
 46 development of new drugs is urgently needed.

47 Bisnaphthalimidopropyl (BNIP) derivatives have previously been shown to possess both  
 48 anticancer activity (7-11) and also have been shown to be antiparasitic against a related  
 49 trypanosomatid *Leishmania infantum* (12-14). The potential activity of three BNIP  
 50 derivatives previously synthesized (10, 11, 15, >96% pure), namely BNIPDiaminobutane  
 51 (BNIPDabut), BNIPDiaminoheptane (BNIPDahep) and BNIPDaminooctane (BNIPDaoct)  
 52 (FIG 1A), against *T. brucei brucei* Lister 427 bloodstream forms (BSF) was investigated.  
 53 These were selected from a series of compounds based on preliminary studies of  
 54 bioavailability and *in vitro* and *in vivo* activity against both *T. brucei* and *L. infantum* (12  
 55 and unpublished data). The *in vitro* antiparasitic activity was assessed using a resazurin  
 56 assay, as previously described, with minor modifications (incubation with  $10^3$   
 57 parasites/well, in 200  $\mu$ L, 16). All three BNIPs demonstrated a potent inhibitory effect on

3

the parasites' growth, with  $IC_{50}$  within the nanomolar range (FIG 1B, TABLE 1). BNIPDabut was the most active compound with an  $IC_{50} \pm SD$  of  $2.4 \pm 1.0$  nM similar to the reference drug pentamidine with  $2.9 \pm 0.7$  nM (TABLE 1). Since this class of compounds has previously been described as inhibitors of the *L. infantum* Silent information regulator 2 related protein 1 (*LiSir2rp1*, accession: AAN39039.1) (15), we evaluated whether inhibition of the *T. brucei* orthologue, *TbSir2rp1* (accession: AAX70528.1) would be a possible mechanism of action. Whereas BNIPDabut was shown to inhibit the  $NAD^+$ -dependent deacetylase activity of *TbSir2rp1* with an  $IC_{50} \pm SD$  of  $155 \pm 42$   $\mu$ M, suggesting that this is not the major mechanism of action (data not shown), *LiSir2rp1* was inhibited with an  $IC_{50} \pm SD$  of  $35.0 \pm 5.8$   $\mu$ M (15). The 47% identity between *LiSir2rp1* and *TbSir2rp1* obtained by protein sequence alignment (Clone Manager 9, BLOSUM 62 scoring matrix) might explain the differences observed (17). Moreover, no correlation between the enzymatic inhibition and activity towards *T. brucei* parasites was observed. To evaluate *in vitro* toxicity towards mammalian cells, all the compounds were studied with the MTT assay (18) in THP1-derived macrophages and two primary cell cultures: rat cortical neurons and mouse hepatocytes (TABLE 2). The  $CC_{50}$  values for these molecules translate into selectivity indexes ( $SI = CC_{50}/IC_{50}$ ) higher than 100. All BNIP derivatives exhibited high SIs, with BNIPDabut in particular being at least 800 times more selective towards *T. brucei* parasites. All BNIPs had potency and selectivity that warranted additional characterization (TABLE 2). To further evaluate the potential toxic effects of BNIPs in host cells, a set of *in vitro* assays was performed in hepatocytes and neuronal primary cells. These assays evaluated different possible mechanisms of toxicity based on:

- a) reactive oxygen species determination (CM-H2DCFDA probe, by High Content

81 Analysis – HCA); b) mitochondrial dysfunction (TMRM probe, by HCA); c) membrane  
82 integrity (lactate dehydrogenase quantification); d) apoptosis (caspase 3/7 activation); e)  
83 either DNA damage for hepatocytes (H2AX antibody, by HCA) or neurite outgrowth for  
84 neurons (anti-tubulin III antibody, by HCA); f) cell viability as measured by WST-8 probe  
85 and g) Hoechst staining for nuclear detection. Nimesulide (400  $\mu$ M) was included as a  
86 positive control and the vehicle as a neutral control (19-21). The relative percentage of  
87 deviation from the neutral control was quantified and assigned with a number from 0 to 5  
88 according to the following criteria: 0 (0-20% deviation), 1 (20-40%), 2 (40-60%), 3 (60-  
89 100%), 4 (100-1000%), or 5 (>1000% deviation). The sum of these values was posteriorly  
90 ranked to create a combined injury criteria that varied from no injury (0), low injury (1 to  
91 <5), moderate injury ( $\geq 5$ -to <12) to high injury ( $\geq 12$ ). All BNIPs showed a dose-dependent  
92 injury score close to pentamidine in both cell types (FIG 2A-B). BNIPDabut had a toxicity  
93 profile indistinguishable from the reference drug pentamidine.

94 To infer metabolic stability, mouse microsomes were incubated over 45 minutes  
95 with 5  $\mu$ M of each compound and the drug was quantified by LC-MS/MS. Similarly to  
96 pentamidine, BNIPDabut was more stable than both BNIPDahep and BNIPDaoc, with 95  
97 to 100% of the drug not being metabolized (TABLE 3). This high metabolic stability is an  
98 indicator that the molecule is not easily subjected to common inactivation or loss of  
99 potency by reactions catalized by liver enzymes, and is kept intact in circulation for longer  
100 periods.

101 To determine the pharmacokinetics of BNIPDabut, a 10 mg/kg dose was  
102 administered to BALB/c mice by intravenous injection. Five minutes after, a concentration  
103 of 58 nM was achieved and during the following 24 hours remained higher than 41 nM  
104 (data not shown), thus approximately 8 times higher than the calculated  $IC_{90}$ .

105 Taking in consideration the previous results, BNIPDabut was chosen for *in vivo* efficacy  
 106 studies. All the experiments involving animals were carried out in accordance with the  
 107 IBMC Animal Ethics Committees and the Portuguese and European Authorities for Animal  
 108 Health guidelines. *T. b. brucei* Lister 427 parasites were transfected with a construct kindly  
 109 provided by M. Taylor, in which the red-shifted luciferase gene (PpyRE9H) is flanked by  
 110 5'VSG/3'tubulin (22). Upon transfection, clones were screened for bioluminescent signal  
 111 and the ones expressing the highest levels were selected. Their *in vitro* growth was  
 112 compared to wild type parasites and found to be similar (data not shown). *In vitro* detection  
 113 limits were also analyzed for BSF in a 96-well plate and determined to be about 2500 cells  
 114 (data not shown). BALB/c female mice were inoculated intraperitoneally with  $10^4$  BSF.  
 115 Three days post-infection, five groups of mice (n=4) were treated intravenously with:  
 116 saline, pentamidine at 2.5 mg/kg/day, DMSO at 16.7%, or BNIPDabut both at a 10  
 117 mg/kg/day and a 20 mg/kg/day dose. Pentamidine was administered for 4 days, while  
 118 BNIPDabut and the respective vehicle (DMSO 16.7%) were administered for 6 days (FIG  
 119 3A). No adverse effects were observable following any administration regimen. Treatment  
 120 efficacy was followed through whole animal live imaging using an IVIS Lumina LT  
 121 (Perkin Elmer). Parasitaemias were also assessed, and animals were euthanized after  
 122 reaching a parasitaemia of  $10^8$  parasites/mL. Similarly to pentamidine, two administrations  
 123 of BNIPDabut both at 10 and 20 mg/kg efficiently reduced parasitaemia below detection  
 124 limit ( $5 \times 10^4$ /mL) (FIG 3B). However, whole mice imaging reveals that reduction of  
 125 bioluminescent signal to the background level (obtained with non-infected mice) is only  
 126 achieved in mice treated with 20 mg/kg of BNIPDabut during at least 5 consecutive days as  
 127 opposed to 2 days treatment for the pentamidine group. Although treatment with 20mg/kg  
 128 BNIPDabut apparently cleared the infection, cure was not achieved as the parasite burden

relapsed when treatment was stopped (FIG 3B). Indeed, BNIPDabut (10 and 20 mg/kg) treatment increases mice survival, but in contrast with pentamidine, animals were not cured (FIG 3C). A hypothesis is that BNIPDabut, although highly trypanocidal cannot reach and clear all parasites, either due to potency, distribution, or both. A frequent observation was that recurrence of parasitaemia was preceded by imaging of parasite loads in the peritoneal zone, where animals were originally injected with the parasites. It has been demonstrated that trypanosomes invade extravascular tissues as a defense mechanism against host immunity, and that this process may be related with relapses after treatment interruption (23, 24). Indeed, the presence of parasites in the extravascular tissues might explain the discrepancy of radiance values between days 4 and 12 while average parasitaemia remain similar in mice treated with BNIPDabut (10 and 20 mg/kg) (FIG 3C). Nonetheless, it remains to be elucidated whether BNIPDabut is active on a mouse model of the late stage of the disease as this is the central objective in drug discovery against human African trypanosomiasis. Additional chemical modifications to BNIPDabut may improve potency and/or distribution of the drug, while maintaining or improving the toxicity and metabolism profile.

In conclusion, this work demonstrates that BNIPDabut has potent *in vitro* and *in vivo* antitrypanosomal activity with acceptable toxicity and high metabolic stability. However, chemical modifications are needed in order to improve its pharmacodynamic and/or pharmacokinetic properties.

#### FUNDING

The research leading to these results has received funding from: the European Community's Seventh Framework Programme under grant agreement No.602773 (Project

KINDReD) and Fundação para a Ciência e Tecnologia (FCT)/Ministério da Educação e  
Ciência (MEC) cofunded by FEDER, partnership agreement PT2020, through the Research  
Unit No.4293.

L.G. was supported by the Fundação para a Ciência e Tecnologia through grant  
SFRH/BD/81604/2011. N.A.G.G. was supported by NMTrypI– PR300103-BD scholarship.

D.M.C. was supported by KINDReD-PR301404-BD III scholarship.

J.T. is an Investigator FCT funded by National funds through FCT and co-funded through  
European Social Fund within the Human Potential Operating Programme.

161

162

#### ACKNOWLEDGEMENTS

Luciferase-expressing plasmid was kindly provided by Martin Taylor, who constructed it  
with the firefly luciferase gene variant *PpyRE9h* from Bruce Branchini Lab.

166

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#### 242 243 **FIGURE LEGENDS** 244

245 **FIG 1** *In vitro* antitrypanosomal activity of BNIP compounds.

246 A) Chemical structures of pentamidine and BNIP derivatives BNIPDabut, BNIPDahep,  
247 BNIPDaoct. B) Growth inhibition curves of *Trypanosoma brucei brucei* BSF incubated *in*  
248 *vitro* with the indicated concentrations of pentamidine, BNIPDabut, BNIPDahep or  
249 BNIPDaoct for 72h. Parasite density was evaluated using resazurin. Dots and error bars  
250 represent the mean + standard deviation of antiparasitic activity. Data of 3 independent  
251 experiments.

252

253 **FIG 2** *In vitro* toxicity of BNIP derivatives.

254 A) Hepatotoxicity injury score. The score was calculated as the sum of individual scores  
255 obtained from a panel of *in vitro* cytotoxicity assays that include: measurement of reactive  
256 oxygen species using CM-H2DCFDA and cells imaging by high content analysis;  
257 assessment of mitochondrial dysfunction measured by TMRM probe dynamics in cells and  
258 image by high content analysis; membrane integrity assayed by lactate dehydrogenase  
259 quantification; DNA damage by imaging with H2AX antibody and high content analysis;  
260 and apoptosis by caspase 3/7 activation; Hoechst staining for nuclear detection; and cell  
261 viability by WST-8 probe. Nimesulide (400  $\mu$ M), an approved drug with a mild  
262 toxicological profile, was included as a toxicity control. Individual scores are calculated  
263 based on the relative percentage of deviation from the negative control quantified and  
264 assigned with a number from 0 to 5 according to the following criteria: 0 (0-20%  
265 deviation), 1 (20-40%), 2 (40-60%), 3 (60-100%), 4 (100-1000%), or 5 (>1000%  
266 deviation). The data represent the mean sum of these values + standard deviation B)  
267 Neurotoxicity Injury Score. The score was calculated similarly to the hepatotoxicity score  
268 but instead of DNA damage by H2AX antibody, an assay to test neurite outgrowth as

12

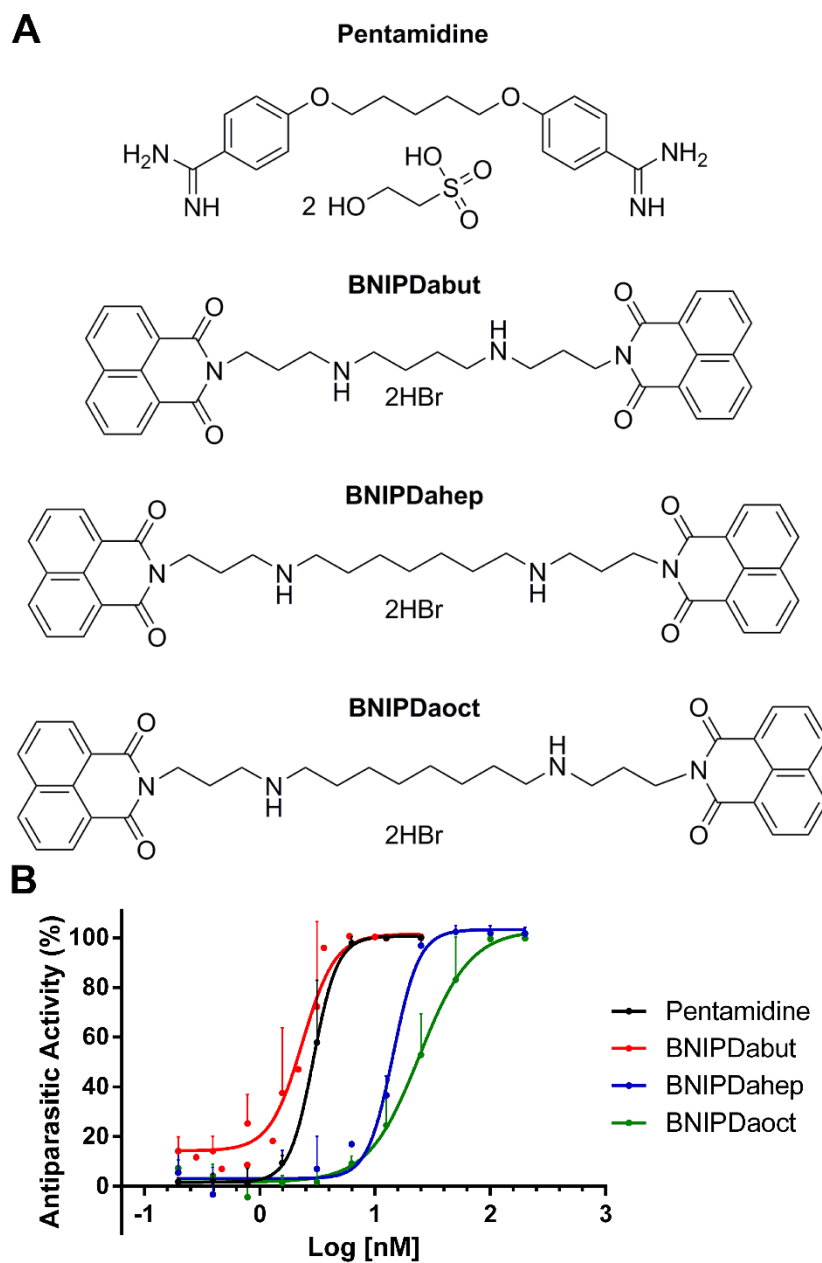
269 imaged with an anti-tubulin III antibody and high content analysis was performed. The data  
 270 represent the mean sum of these values + standard deviation. Data of 2 independent  
 271 experiments.

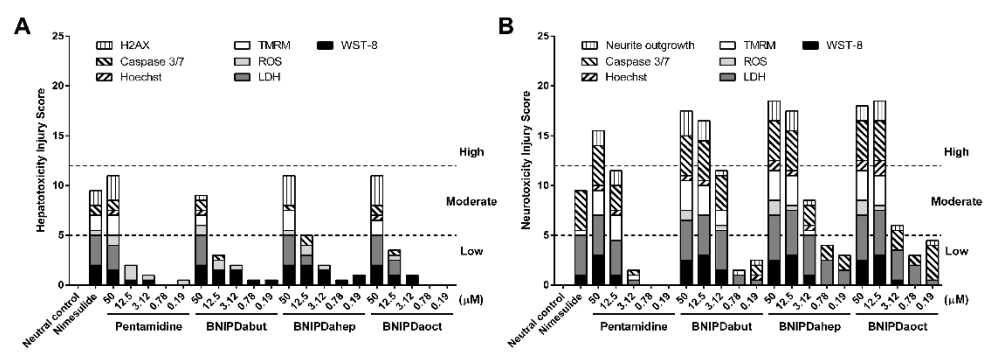
272

273 **FIG 3** BNIPDabut *in vivo* efficacy against *T. b. brucei*.

274 A) Schematic of the experimental design to evaluate the *in vivo* efficacy of BNIPDabut. B)  
 275 Mice were infected with  $10^4$  LUC<sup>+</sup> BSF by intraperitoneal injection and initiated the  
 276 different treatments 3 days post-infection. Whole mice bioluminescence imaging was done  
 277 at days 3, 4, 9 and 12 using an IVIS LUMINA LT and upon injection of 2.1mg luciferin.  
 278 Bioluminescence average radiance (p/sec/cm<sup>2</sup>/sr) of whole mice was quantified and the  
 279 mean + standard deviation (n=4) is shown in bars. Parasitemia was determined using a  
 280 haemocytometer and the mean + standard deviation is represented by red dots. Red crosses  
 281 represent the parasitaemia of the only animal where parasites could be detected and  
 282 quantified. Parasitaemia detection limit is  $5 \times 10^4$  parasites/mL. C) Kaplan-Meier survival  
 283 curves of the infected mice treated with controls and experimental doses of BNIPDabut. B-  
 284 C) The data is representative of 3 independent experiments.

285





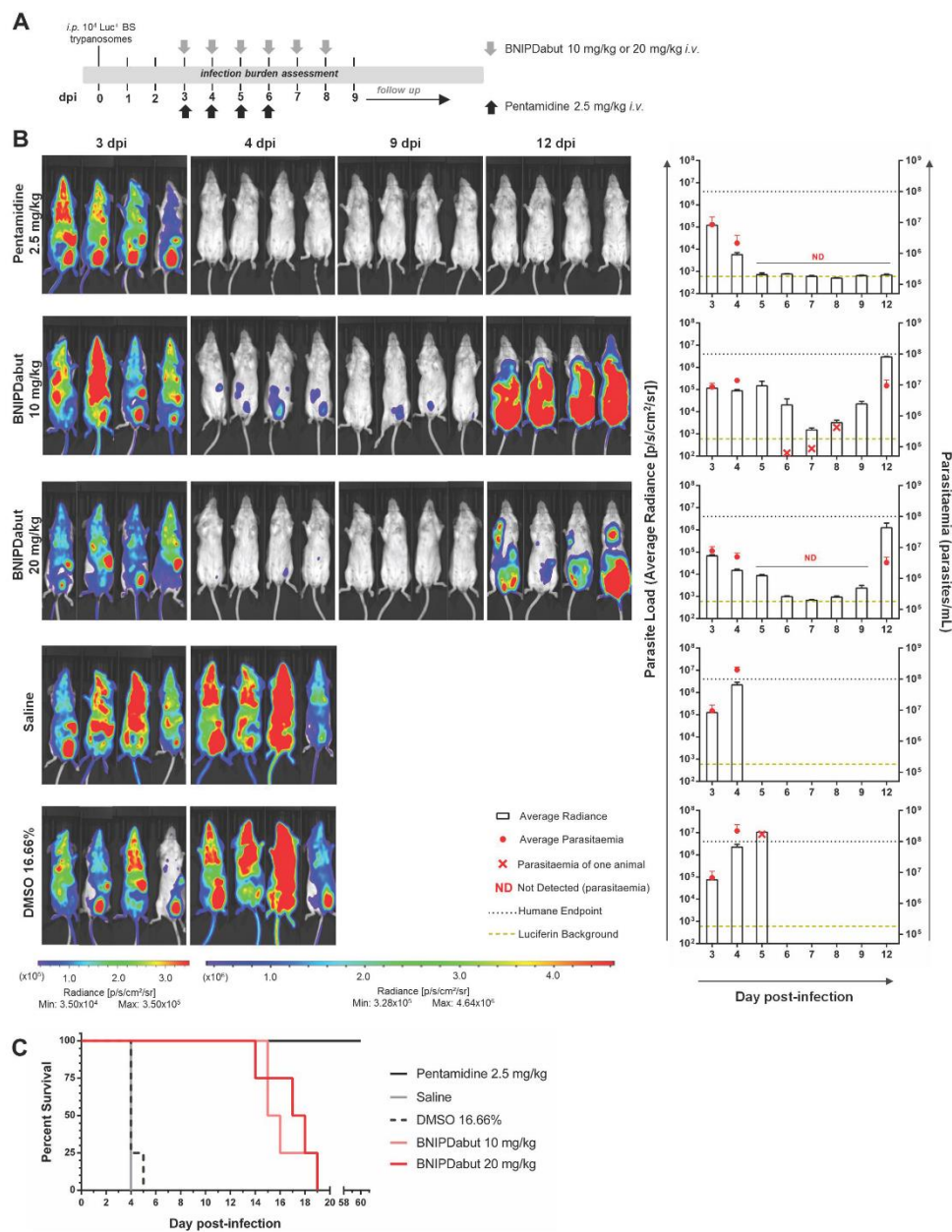




TABLE 1 *In vitro* activity of BNIP derivatives against *Trypanosoma brucei* 1.427 bloodstream forms

	IC <sub>50</sub> ± SD (nM)	IC <sub>50</sub> ± SD (nM)
Pentamidine	2.94 ± 0.74	5.26 ± 0.58
BNIPDabut	2.35 ± 0.99	3.83 ± 1.40
BNIPDahop	14.32 ± 1.21	23.07 ± 1.01
BNIPDaoct	26.15 ± 10.43	63.36 ± 21.19

TABLE 2 *In vitro* cytotoxicity of BNIP derivatives in different cell types

	CC <sub>50</sub> ± SD (μM)			Selectivity Index*		
	THP1	Hepatocytes	Neurons	THP1	Hepatocytes	Neurons
Pentamidine	47.73 ± 3.32	18.21 ± 0.66	8.23 ± 0.88	16259	6203	2803
BNIPDabut	5.90 ± 0.40	9.19 ± 0.06	2.06 ± 1.69	2514	3916	878
BNIPDahep	3.34 ± 0.11	4.23 ± 0.48	2.31 ± 1.65	233	295	161
BNIPDaocet	3.88 ± 0.59	18.35 ± 4.58	3.97 ± 1.30	148	702	152

\*Selectivity Index = CC<sub>50</sub> cell line/CC<sub>50</sub> *T. brucei*

TABLE 3 Mouse microsomal stability

	Metabolic stability (%)	<i>In vitro</i> Intrinsic Clearance $CL_{int}$ ( $\mu$ L/min/mg protein)	Degradation non NADPH-dependent (%)
Pentamidine	95 - 100	-	< 5
BNIPDabut	95 - 100	-	< 5
BNIPDahep	85	7	< 5
BNIPDaect	64	20	9



# **PART V**

## Discussion and conclusions



### 5.1. Trypanosomiasis disease control: the missing piece

Despite the efforts of many individuals and organizations over the years, human trypanosomiasis remains one of the most neglected diseases in the world. Chagas disease in particular, is a leading cause of disease and disability in Latin America, with thousands of deaths every year [66]. The negligence is particularly patented by the lack of new drugs. Indeed, the available treatment options, benznidazole and nifurtimox, were discovered more than 40 years ago. Different strategies have been tried to control the disease, but the most impactful so far has been the control of the transmitting vector led by WHO and PAHO. Vectorial control has caused the reduction in cases from a staggering 24 million in the 1980s to about 6 million nowadays [66, 174], with some countries considered to be free of domestic vectorial transmission. Continued and rigorous implementation of the disinfestation programs in the remaining zones should decrease even further the global numbers of *T. cruzi* vectorial transmission. Also, the screening of donor blood and transplant organs now widely implemented in the endemic regions and other parts of the world have greatly reduced the number of cases transmitted by this route. Whereas the global interruption of the domestic cycle will be a major breakthrough and reduce to a minimum the number of new cases of Chagas disease, complete eradication of the parasite, however, is unlikely to be achieved due to the huge natural reservoir of *T. cruzi* and the many species of triatomines capable of transmission in the sylvatic environment [92, 99, 100].

What then, stands in the way of disease control for Chagas? First, no major technological advances are required to interrupt vectorial transmission responsible for the majority of new cases; second, decades of research in the molecular mechanism of *T. cruzi* biology, the particularities of pathogenesis of the disease or the dynamics of immune response against the parasite have failed to translate into therapeutic alternatives; and finally, vaccination, either preventive or therapeutic, has remained an elusive achievement. The answer lies in a new drug. A new, safer, cheap, easy to administer and efficacious drug that is able to treat not only new cases, but also the millions of people already affected by the chronic stage and indeterminate form of the disease. There is growing evidence that chronic manifestations are ultimately related with inflammation resulting from parasite persistence [355, 356] and effective treatment of these cases would be highly beneficial to stop the development of symptomatology.

Active drug discovery efforts for Chagas disease have been restricted, until some years ago, to just a very limited number of groups, mostly based in academia. As a consequence, results have been sporadic, slow, ineffective and highly dependent on intermittent funding, failing to deliver an alternative treatment. Chagas disease is as much

neglected by the pharmaceutical industry as it is by research funding agencies, whose majority of funds are directed to research in diseases affecting developed world populations.

Only recently has drug discovery for Chagas been met with concerted, focused efforts. While still not privately embraced by pharmaceutical companies, public-private partnerships have been set up with the objective of bringing together the biology expertise from academia and the technical expertise, facilities and resources from pharmaceutical industries. One organization that is leading the effort to find new therapies for neglected diseases, including human trypanosomiasis, is the DNDi, that has been involved in coordinating activities from early drug discovery to the launch and conclusion of clinical trials for some candidates like inhibitors of ergosterol biosynthesis of *T. cruzi*. The Bill and Melinda Gates Foundation, a non-governmental organization devoted to human development in underdeveloped countries, has recently sign up the challenge of controlling neglected diseases by signing The London Declaration on Neglected Diseases together with the WHO, the World Bank and 13 leading pharmaceutical industries. The Declaration states that by 2020 the signers will achieve, among other ambitious milestones, the eradication of Human African trypanosomiasis (HAT) and the control of American trypanosomiasis. Since then, other initiatives have been launched with the objective of boosting the research in new drugs for trypanosomal diseases, like the European Commission Seventh Framework Program (FP7) consortia NMTrypI and KINDReD. The recent award of the Nobel Prize in Physiology or Medicine 2015 to William C. Campbell, Satoshi Omura and Youyou Tu is a recognition of the importance of drug discovery for parasitosis and should further increase the awareness of neglected diseases by the international community.

Even with the recent involvement of new players and an increase of the scarce research funding, many challenges remain to what are the most effective strategies and models in drug discovery for trypanosomes. This thesis attempted to contribute with some insights to these challenges.

## **5.2. Phenotypic-based drug discovery: a viable approach**

Pharmaceutical research and drug discovery for infectious disease has historically began with what would be classified today as phenotypic assays, and can be traced to the pioneering work of Paul Ehrlich in the 19<sup>th</sup> century while testing the effect of different dyes in trypanosomes [357]. Cultures of the microorganism of interest, be they a bacteria or a parasite, were incubated with a compound of interest, and the selective staining of the dyes



was monitored by microscopy. Products of such “dye therapy” approach were in the origin of well-known chemicals like the crystal violet dye, that was used in blood banks of endemic areas to kill *T. cruzi* parasites present in transfusion blood as a way to reduce transmission by this route [358]. Another example is trypan blue, that is still widely used as a cell biology reagent and that was the starting point for the design of the colorless analogue suramin, a drug still in use today for the treatment of HAT [359]. Such early whole cells drug screening principles were also central to the development of nifurtimox and benznidazole in the 1960s and 1970s, by the pharmaceutical companies Bayer and Roche, respectively [352].

With the genomic era there was a dramatic shift in the way new drugs were discovered. The past 20 years have witnessed incredible advancements in genomics, proteomics, structural biology, computational chemistry and structure based drug-design, that coupled with high-throughput screening and combinatorial chemistry have helped shape the reductionist mentality “one gene – one protein – one drug” [360]. However, the complexity of many diseases and the capacity of adaptation to adverse conditions, like the presence of a xenobiotic evidenced by many living cells, have brought the more naïve phenotypic whole-cell screening strategies back to the spotlight. With modern phenotypic approaches, the effect of a pure molecule in a fully intact whole living organism, bacteria, parasites or human-derived cell lines, results in the identification of hit compounds that are potentially more useful as scaffolds for medicinal chemistry optimization.

While early phenotypic screenings for *T. cruzi* sometimes used the insect specific epimastigote stage due to its extracellular nature and easy of culture, the use of reporter genes expressed during the clinically relevant stage of the disease, the intracellular amastigotes, has met a widespread application. The first of such assays was based on the  $\beta$ -galactosidase-expressing parasites that made possible the detection of anti-*T. cruzi* activity by a colorimetric reaction [361]. Later, tdt-tomato and luciferase genes were also constitutively expressed in parasites, which allowed the more sensitive measurement of a fluorescent or luminescent signal, respectively [362].

However, the use of genetically unmodified parasites has always been an attractive pursuit, made available only recently due to technologic advancements. One such assay was developed by researchers at Institut Pasteur Korea and used in part of this thesis work (section 4.2.1.1). This cell-based assay employed the use of wild type parasites of *T. cruzi* infecting a non-modified cell line and the imaging of the resulting infection (in the amastigote stage) by high-content analysis (HCA) microscopy. Furthermore, the assay was developed in the 384-well format, allowing a high throughput testing of compounds. Preliminary cell toxicity is concomitantly determined by quantifying the ratio of host cell nuclei, a clear advantage since it reduces the need of an independent assay to access this parameter.

Statistical validation demonstrated it as a robust assay suitable for drug screening, as assessed by the z-factor of 0.81 [363]. Using this screening assay, a targeted library of 4000 kinase/phosphatase-like inhibitors was screened and allowed the identification of 11 compounds with strong antiparasitic activity and selectivity, suitable for follow up hit-to-lead optimization. In addition, a complementary assay developed for phenotypic profiling also allowed the identification of several compounds that interfered with the development and intracellular differentiation of *T. cruzi*. Compounds that hindered the differentiation from trypomastigotes to amastigote and the replication of amastigotes inside host cells are among the examples of “phenotypic” hits discovered. Because of the complex genetics and still many unknown aspects of *T. cruzi* biology, this type of compounds has the potential to constitute important chemical genomic tools that may help answering questions like: what triggers stage differentiation and what are the pathways involved? What factors are responsible for parasite persistence? How are amastigotes kept dormant for years to decades in host cells, hidden from the immune system? Due to the nature of the chemical library, it is likely that the compounds target kinases, of which the *T. cruzi* genome has 190 annotated members [19]. *T. cruzi* and other trypanosomatids have a relatively big kinome when compared with other parasites that undergo several stage differentiation and contact with distinct environments like *Plasmodium* spp. [364, 365]. One hypothesis is that while in metazoa and yeast the ultimate targets of many signaling cascades are transcriptions factors which then trigger the expression of new sets of genes, trypanosomatids have constitutive transcription of a majority of genes in large polycistronic units, hinting at a greater role of PTMs like phosphorylation. The precise mechanism of action, however, will only be evaluated by target deconvolution. Another application of such phenotypic assays is the preliminary profiling and evaluation of the mechanism of action for established or newly discovered drugs for *T. cruzi*, for instance, as trypanocidal or trypanostatic agents.

HCA assays are also valuable for the study of drugs already in use for the treatment of *T. cruzi*, such as nifurtimox (section 4.2.1.2). This drug, effective in the acute stage of the disease but possessing many side-effects that often lead to treatment interruption [352], is synthesized as a racemic mixture of R- and S-enantiomers. It was hypothesized that one of the enantiomers would be majorly contributing or solely responsible for toxicity, activity, or other pharmacokinetic and pharmacodynamic property of the medicine that might justify its use as a purified isomer. By using this assay on different strains (at least one representative of each discrete typing unit - DTU [366]), it was possible to verify that the sensitivity to nifurtimox varied among different genotypes, but individual genotypes had the same sensitivity to each individualized R- and S-enantiomer, as well as to the racemic mixture [367]. This study also allowed to conclude that the enantiomers were similar in what regards

to their *in vitro* cytotoxicity, physicochemical properties, *in vitro* metabolic stability and *in vivo* efficacy and pharmacokinetics [367]. As such, there is no benefit in separation of the racemic mixture.

Two distinct families of compounds were also evaluated by an equivalent assay designed with C2C12 cardiomyocytes as host cells (section 4.2.1.3 and 4.2.1.4). A set of newly synthesized 7-aryltioetherthieno[3,2-b]pyridine analogues demonstrated to have some antiparasitic activity against trypanosomes, mostly *T. brucei* bloodstream forms and also with some analogues being active against *T. cruzi* amastigotes. However, cell toxicity NOAELs were similar to the dose of demonstrated antiparasitic activity, suggesting selectivity was reduced. A group of naphthalimide derivatives was also evaluated for their potential antiparasitic activity against *T. brucei* and *T. cruzi*. The majority of the compounds were inactive towards *T. cruzi* at 10  $\mu$ M, with exceptions found in the subset of derivatives functionalized with a urea group. *T. brucei* bloodstream forms were more sensitive to all the groups of substituted naphthalimide derivatives, probably a consequence of their extracellular nature. Once more, however, host cell cytotoxicity precluded a high selectivity.

Finally, specifically developed software allows the evaluation of not only compounds potency ( $IC_{50}$ ), but also efficacy (parasite clearance)[368]. A recent study comparing efficacy and potency of distinct drugs in use or undergoing clinical trials for Chagas disease with strains representative of different DTUs found that nitroheterocyclic compounds like benznidazole induced 100% of parasite killing contrary to posaconazole or ravuconazole, despite having an  $IC_{50}$  around 1000 times lower [369].

The major disadvantages related with this screening method are the high costs of the technology that are not accessible to all laboratories, and the need of bioinformatics for the design and validation of the image analysis software/plugins.

### **5.3. Target-based drug discovery: is Sir2 a potential drug target in *Trypanosoma cruzi*?**

Another strategy that can be employed in drug discovery is the target-based approach. In this case, the process begins and is centered on a particular molecular target. One family of proteins that has gained increased interest as potential drug targets against parasitic diseases is that of sirtuins [370, 371]. The hypothesis arose by the time that it was demonstrated that sirtuins are life-span regulators in organisms like yeast, flies and worms [231, 372, 373]. Many groups promptly investigate whether sirtuin orthologues were present in the genome of pathogenic organisms and if they also have important functions that could

be exploited for therapeutic applications. A sirtuin from *T. brucei* was the first trypanosomal sirtuin to be described and evaluated as a drug target. Although the protein had a clear NAD<sup>+</sup>-dependent deacetylase activity, in addition to ADP-ribosylation activity, and was important for the protection of DNA from damage caused by alkylating agents, RNAi experiments did not prove essentiality for *T. brucei* [317]. Later, another lab studying the same protein in bloodstream forms also reported that, in addition to the DNA protective role of Sir2rp1 and the participation in telomere gene silencing, the protein was not essential for the parasite, as demonstrated by both RNAi and gene knockout experiments [322]. In addition, the other two paralogues in *T. brucei*, TbSir2rp2 and TbSir2rp3 were also not essential for parasite survival [322]. Studies in *L. infantum* Sir2rp1, however, have disclosed an important role for the protein in *in vitro* and *in vivo* infections of the parasite. Double knockouts for the gene were never obtained, and both copies of the endogenous allele were only possible to delete when an ectopic copy was provided, thus strongly suggesting essentiality of the protein [308]. While *L. infantum* Sir2rp1 single knockouts did not have apparent defects on promastigotes replication, the multiplication of axenic and intracellular amastigotes was dramatically affected [308]. Furthermore, single knockouts failed to establish an infection in mice like the wild type parasites [308]. Over the course of the studies leading to this thesis, two publications reported a role of TcSir2rp1 and TcSir2rp3 in the epimastigote replication, metacyclogenesis, infectivity and amastigote replication in *T. cruzi* parasites, mounting up evidence that these proteins have important functions in different life-cycle stages [218, 219].

One important aspect for the viability of targeting sirtuins in parasites is the homology between the protein of interest and other proteins present in the host organisms. Although sirtuins are conserved through evolution [194], significant difference at the sequence level can be found between trypanosomatid and human homologues. For instance, *T. cruzi* Sir2rp1 shares only 33% identity with mammalian SIRT2, its closest homologue (Multi-way protein alignment, BLOSUM 62) [374].

Another argument that has led to the consideration of a *T. cruzi* sirtuin as a drug target is that this family of proteins is considered to possess structural properties adequate to inhibition by small-molecule compounds. In particular, the catalytic site is located inside a hydrophobic channel formed at the interface between the two constituting domains, the Rossmann fold containing the NAD<sup>+</sup> binding domain and the Zn<sup>2+</sup> ion binding domain [215]. Catalytic pockets buried inside the protein are considered an essential feature for target druggability [375].

One last fact that prompted the evaluation of TcSir2rp1 as a drug target was the previous evidence that a class of experimental compounds preferentially inhibited LiSir2rp1

over the human homologue SIRT1 [376]. The synthesis of selective human sirtuin inhibitors has been successfully achieved based on structural knowledge of the catalytic site, like it has been demonstrated for SIRT2 [377, 378].

The first steps of the target-based drug discovery strategy were focused on the generation of knockout and overexpressing mutants for the Sir2rp1 gene in *T. cruzi* (section 6.1). At the date of writing this thesis, all attempts at obtaining mutant parasites were unsuccessful and further efforts to their achievement are currently underway.

In parallel to the time intensive genetic manipulation of *T. cruzi*, it was decided to proceed with the biochemical characterization of the molecular target. Amplification of the gene from CL Brener strain and its heterologous expression in *E. coli* as a recombinant protein produced an active sirtuin with NAD<sup>+</sup>-dependent deacetylase activity. In addition, it is mostly insensitive to TSA inhibition, allowing the classification as a canonical sirtuin belonging to the class III histones deacetylases. The kinetic characterization identified TcSir2rp1 as a *bona fide* sirtuin with robust deacetylation activity similar to the founding member yeast Sir2 [200]. The kinetic parameters  $K_m$  and  $k_{cat}$  of TcSir2rp1 for NAD<sup>+</sup> were found to be highly similar to the ones of TbSir2rp1, despite just a 61% identity between them, suggesting structure conservation [325].

Enzymatic inhibition by small molecule compounds, an essential step in druggability assessment of novel therapeutic targets, was evaluated by nicotinamide, a classic non-competitive inhibitor of sirtuins. TcSir2rp1 was inhibited by nicotinamide, albeit at a relatively high IC<sub>50</sub> when compared with other sirtuins (4-fold higher for hSIRT1 and 11-fold higher for LiSir2rp1) [376]. Different nicotinamide sensitivities are found among distinct sirtuins, and may explain the differences described [378]. Nicotinamide inhibits deacetylation by binding to a conserved C pocket present in sirtuins that participates in NAD<sup>+</sup> binding and catalysis, where it promotes a base-exchange reaction at the expense of deacetylation [379]. A hypothesis for the high IC<sub>50</sub> for nicotinamide in TcSir2rp1 could be related with structural characteristics of this conserved C pocket. Structural determination of TcSir2rp1 by X-Ray crystallography currently ongoing in our group will certainly highlight these differences. Contrary to previous studies [380], we could not observe any antiparasitic activity of nicotinamide against *T. cruzi* amastigotes for up to a concentration of 2 mM. Several studies report the activity of nicotinamide against parasitic protozoa [381-383], but to our knowledge, none clearly establishes a relation between antiparasitic activity and sirtuin inhibition.

Other biochemical functions and protein interactions have been attributed to Sir2rp1 in related trypanosomatids, and future experiments should shed light whether it applies to TcSir2rp1. One of the biochemical functions that has been characterized for both TbSir2rp1

and LiSir2rp1 is ADP-ribosylation. Both orthologous showed to ADP-ribosylate calf thymus histones and BSA [317, 384]. Later studies involving TbSir2rp1 demonstrated that this biochemical function is dependent on acetylated histones, is coupled to the deacetylase activity of the sirtuin but occurs at a much lower rate than the latter [325]. In fact, even though ADP-ribosylation has clear functions in both physiological and pathogenic situations when catalyzed by other ADP-ribosyltransferases [384, 385], the reaction catalyzed by sirtuins is currently challenged to be an unspecific side-reaction [321].

Like the human SIRT2, *L. infantum* Sir2rp1 was also found to be associated with tubulin [309], the major component of trypanosomatids cytoskeleton formed by an array of subpellicular microtubules that span the whole cell of the parasite [53]. SIRT2 is a tubulin deacetylase that displays a higher affinity for tubulin than for histones [193], and has been found to be linked to regulation of mitotic progression [247], chromatin condensation [386] and cell migration [387]. TcSir2rp1 overexpression in *T. cruzi* was found to increase the deacetylation level of endogenous tubulin [218]. It is interesting to note that Sir2rp1 from *T. cruzi* is a cytoplasmic protein like LiSir2rp1 and not nuclear like *T. brucei* Sir2rp1. Since *T. cruzi* shares some characteristics with *L. infantum* like the amastigote intracellular stage, it should not be ruled out that Sir2rp1 may have functions in the cytoskeleton remodeling necessary for stage differentiation. Several proteins, sirtuins included, have demonstrated the ability to shuttle from the nucleus to the cytoplasm and vice-versa [388, 389]. SIRT2, the closest sirtuin homologue of mammalian cells, is actively exported to the cytoplasm during interphase, but is accumulated in the nucleus from prophase until cytokinesis where it co-localizes with important mitotic structures like centrosomes and the mitotic spindle [390]. Curiously, analysis of TcSir2rp1 by Wregex and cNLS Mapper, bio-computational tools that identify nuclear export signals (NES) and nuclear localization signals (NLS), respectively, indicate the presence of non-canonical NES/NLS in the sequence of this sirtuin [391, 392]. Whether TcSir2rp1 does shuttle to nucleus during particular moments of *T. cruzi* life cycle, for instance to repair DNA damage like the *T. brucei* orthologue, remains to be reported.

In conclusion, biological relevance of other biochemical and cellular roles of TcSir2rp1 besides deacetylase activity shall be properly elucidated once mutants are generated.

#### **5.4. Naphthalimide derivatives: sirtuin inhibitors with trypanocidal activity**

Naphthalimides are a class of compounds that have generated intense interest as active molecules with potential to treat a range of conditions [393]. A naphthalene ring linked to an imide group that forms a third heterocycle composes the basic chemical structure of naphthalimide derivatives. This moiety has a planar nature and is considered to be responsible for the pharmacological activities attributed to compounds derived from this structure, that can be as distinct as anticancer, antibacterial, antiprotozoal, antiviral, analgesic, and anesthetic [393]. Their potential as anticancer compounds has received particular attention, mostly because of their DNA intercalating properties and also to their reported activity as topoisomerase inhibitors [394-396]. Compounds like amonafide and bisnafide have been proposed as anticancer agents and have inclusively reached clinical trials in the past [397, 398]. Elinafide is another derivative with two naphthalimide moieties that has been evaluated in preclinical studies and demonstrated potential against various mouse xenograft models [399]. This last compound was in the origin of the synthesis of the first BNIPs that differed in the alkyl chain linking the naphthalimide and amine group - a propyl instead of an ethyl chain [400]. These derivatives showed potential activity against breast cancer MCF-7 cell line and actively bound DNA as demonstrated by thermal denaturation measurements, ethidium bromide displacement and DNA gel mobility [400]. Later derivatives that varied in the length of the chain linking the two amines of bisnaphthalimidopropyl groups were also evaluated against cancer cell lines and promastigotes of the parasite *L. infantum* [401]. While screening for enzymatic inhibitors of the recently characterized LiSir2rp1, BNIPs were identified as inhibitors of its deacetylase activity [376]. Furthermore, they were active against intracellular amastigotes, the clinically relevant stage of the parasite present in humans, at concentrations in the single micromolar range [376].

The promising results against *L. infantum* led to the testing of BNIPs as inhibitors of the related trypanosomatid *T. brucei* and its Sir2rp1 orthologue, TbSir2rp1 (section 4.2.2.1). BNIPs revealed to be very potent inhibitors of *in vitro* parasite growth, with one of the compounds, BNIPDabut with an  $IC_{50}$  in the range of the reference drug pentamidine. However, when tested against the TbSir2rp1 recombinant enzyme, BNIPDabut had an  $IC_{50}$  more than  $10^4$  times superior to the  $IC_{50}$  against the whole cell parasite, indicating that Sir2rp1 inhibition is probably not a major mechanism of action for the compound. Whether BNIPDabut inhibits other *T. brucei* sirtuin enzymes remains to be elucidated. It should be noted that RNAi and gene knockout experiments of the three sirtuins did not led to a deleterious effect, and unlike LiSir2rp1, there is no indication of essentiality of these proteins [317, 322]. The optimal *in vitro* properties of BNIPDabut led to the testing with an *in vivo* model of trypanosomiasis by bioluminescence imaging. Although BNIPDabut maintained a

strong trypanocidal activity *in vivo*, as assessed by the decrease in bioluminescent signals to levels similar to those of the reference drug control pentamidine, it was not sufficient for infection clearance, as animals' parasitaemias relapsed shortly after treatment interruption. Nevertheless, BNIPDabut should constitute a scaffold for further consideration in HAT drug discovery.

BNIP derivatives were also tested against TcSir2rp1 and *in vitro* cultures of *T. cruzi* amastigotes (section 4.2.2.1). BNIPs demonstrated to inhibit the deacetylase activity of this enzyme, with BNIPSpd as the most potent compound, showing a dose-dependent effect on inhibition. BNIPSpd also proved to be active and selective against amastigotes of *T. cruzi* in a HCS assay. In this work, a new set of derivatives was also synthesized in order to improve both solubility and binding to cellular targets, mostly by including cyclic structures and heteroatoms in the carbon chain linking the two naphthalimide groups. The most active compounds were BCNIPP, also a TcSir2rp1 inhibitor, and trans BNIP-1,4-Dacyhex, a derivative of BNIPDabut that weakly inhibited the enzyme. In turn, BNIPDabut had some inhibitory activity on *T. cruzi* amastigotes, with low selectivity, but also didn't inhibit TcSir2rp1 at 10  $\mu$ M. The activity of BNIPSpd in a mice model of Chagas disease using bioluminescent parasites was also determined and found to be inexistent at the doses tested. An explanation might be the poor pharmacokinetic profile of the compounds, which fails to ever achieve at least the *in vitro* IC<sub>50</sub> against *T. cruzi* amastigotes.

Altogether, our data indicate that BNIP derivatives may not be acting entirely by a mechanism of Sir2rp1 inhibition, with other targets contributing to the activity detected. BNIPs were originally designed and developed as anti-cancer agents [402, 403] by a mechanism of DNA intercalation. This property might explain some of the cytotoxic effects verified against host cells, but may also be an important mechanism of activity towards the parasite, especially since trypanosomes are highly susceptible to intercalating agents [404]. The activity of mono naphthalimidopropyl derivatives was also assessed against *T. brucei* and *T. cruzi* parasites, but they were comparatively less active against the parasites, suggesting that a second naphthalimidopropyl group is essential for potency.

Confirmation of the mechanism of action can be undertaken by appropriate target deconvolution experiments [405]. The most common are biochemical methods that employ some variation of biochemical affinity purification, where the compounds are immobilized in a column, and allowed to contact with protein extracts, preferably previously fractionated. After stringent washing steps, the bound proteins are eluted and identified. Such strategy has been employed in the identification of small molecule activators of cryptochrome of mammalian cells [406]. A disadvantage is that there is a bias towards high affinity ligands, and when the target is relatively less abundant or has less affinity, important targets may



not be detected. Furthermore, the washing steps may eliminate protein complexes that may be important for appropriate drug activity.

Genetic methods can also be valuable for target deconvolution. Gene knockouts and RNAi screens can be used to try to phenocopy a compound's effect [407]. Furthermore, if the mutant is hypersensitive to the compound in question, the evidence that the protein could be the target for the compounds would be strengthened. The validation of trypanothione synthetase and N-myristoyltransferase as drug targets against trypanosomes are examples where the differential sensitivity of an inhibitor in wild type, overexpression, and knockout mutants is clearly illustrated [408, 409]. An additional genetic strategy is based on the generation of resistant cell lines by culturing the parasites in increasing sub-lethal drug concentrations that are posteriorly sequenced to find mutated genes related to the mechanism of action for the compound [407]. Genetic methods have recently been employed in the search of the mechanism of action of oxaboroles [410], a class of compounds in development for HAT, but also active against *T. cruzi* [411, 412].

Chemical genomics can also be applied to the discovery of novel drug targets, as exemplified by the recent characterization of cytochrome b from *T. cruzi*. This enzyme was demonstrated to be selectively targeted in relation to the human homologue by a hit compound coming from a phenotypic screening [413].

### **5.5. Phenotypic drug discovery versus target-based drug discovery: which strategy comes ahead?**

Drug discovery and development is a risky, costly and long endeavor [414]. Early 2000s estimates put the price tag on a new drug around 800 million dollars, but that value has since increased at least two-fold [415]. It can take 12 years on average for a new entity to enter the market and only about 1 in every 10 compounds that enter clinical trials are eventually released [414]. Including the early drug discovery efforts, only 1 in about 50 programs are successful [414]. Furthermore, despite the fact that neglected tropical diseases affect more than 1 billion people worldwide [416], only 0.3% of the funds spent in pharmaceutical industry research and development are allocated to these diseases [417].

The selection of the early drug discovery strategy is then of the utmost importance, especially in an underfunded area. Phenotypic and target-based approaches have long been the two main strategies to obtain the early scaffolds (hits and leads) that are the starting points of the to-be drug. Each strategy has its advantages and disadvantages, and

the focus on one of them will have to be adapted to each particular disease, the tools available and the expertise of the team.

Target-based screening approaches are advantageous in that they are driven by specific hypothesis that take advantage of advancements in genome biology and molecular tools. The strategy is focused on a certain gene or pathway that has previously been characterized and is properly understood. Furthermore, this strategy can directly benefit from structural biology and computational screening and drug design as valuable discovery tools. Finally, because screening of compounds is most of the times based on enzymatic assays, they are amenable to high throughput screening [360].

On the other hand, target-based screening can also have some disadvantages, like the focus on a relatively small biological environment, reproduced in artificial *in vitro* conditions that neglects the complex intracellular environment and the multitude of interactions and interferences that may take place. Also, there are some challenges in what regards the translation to an *in vivo* disease state, where additional complexity may render the target irrelevant for modulation. In addition, there is the possibility of *in vivo* compensatory mechanisms that may null the effect of action at a specific target [360].

Phenotypic strategies are an un-biased approach, allowing the interrogation of complex biological systems as a whole. They have the advantage of being target-free, which may translate better into activity in a human disease state. Beneficial drug properties like cell permeability and no resistance to efflux pumps ensure that the compound reaches its molecular target in the cell. Potential toxicity of the hit compounds can also in principal be detected earlier than with hits coming from target-based screenings [360]. Some disadvantages of the phenotypic strategies are the unknown mechanism of action of the compounds that requires appropriate target deconvolution studies. This aspect may hinder lead optimization, which most times rely on some knowledge about the molecular target. The optimization may be further complicated by the potential effect of the compound in several molecular targets. Since most assays require cell culture, the throughput may be potentially lower. However, recent developments in high-throughput technology have allowed phenotypic assays to catch up with target-based screening [360].

This thesis employed both approaches to the early drug discovery for *T. cruzi*. The target-based approach sought the validation of a protein, TcSir2rp1 by both genetic and chemical validation. The genetic validation of targets for *T. cruzi* has been hindered by the particular resistance to genetic manipulation by the parasite [418]. Successful genetic validation of a molecular target is affected by a number of characteristic, like the availability of inducible and non-inducible expression vectors, the number of drug resistance markers characterized and genetic and physiologic properties of the organism in question [419].

Important feature to consider for a successful validation are: the number of gene copies, the ploidy of the organism, the ease of transfection and *in vitro* culture, the susceptibility to drug selection and the occurrence of sexual recombination [419]. The molecular toolbox for *T. cruzi* lags beyond what is available for other trypanosomes, like *T. brucei*, and has been slowly enriched overtime with advances like overexpression vectors [420], a tet-regulated inducible vector [421], and adaptation to gateway technology [422, 423]. Any information regarding the essentiality of a given protein to the biology of *T. cruzi* has therefore relied in time-consuming and inefficient traditional gene knockout methods [418]. These limitations were reflected in the difficulties in obtaining gene knockouts undertaken during this thesis. One of the examples was the generation of hygromycin resistant parasites when one of the Sir2rp1 deletion constructs was transfected as a circular DNA molecule, but not when the same construct was transfected as a linear molecule with the objective of gene replacement (section 6.1).

Gene knockouts can be achieved by gene disruption, when the coding sequence is interrupted by exogenous DNA and leading to an unnatural transcript and dysfunctional protein, or by gene replacement, that is considered a cleaner approach due to the absence of partially active or toxic proteins/peptides [424]. When a gene is successfully knocked out, care must be taken as to draw the appropriate conclusions: the lack of essentiality must be only considered as to that particular stage and particular culturing conditions [419]. This is especially true for *T. cruzi*, since the life-cycle stage commonly genetically manipulated is the epimastigote, characteristic of the insect vector and not present in disease context. If a strategy fails to achieve or evade gene replacement, then the gene may be considered as essential. Events like insertion of the sequence in another part of the genome, increase in chromosome number or compensatory genetic mutations have all been described to occur in trypanosomatids and are an indication of essentiality [425]. Nevertheless, confirmation should be undertaken by nutritional or genetic rescue [426, 427]. Newer technologies like CRISPR/Cas9 gene editing have recently been applied to the genetic manipulation of *T. cruzi*, and should bring some advancements in this area [58, 428].

On the other hand, chemical validation has been the most exploited target validation strategy for Chagas disease. Some examples are the targets cruzain and CYP51 that have had inhibitors tested in preclinical and clinical trials [173, 429]. In the basis of this approach is the specific inhibition of a target by a small molecule that is later evaluated as to their potential in inhibiting the growth or cause the death of the parasite. Using this strategy, the compounds selected address some druggability issues, like cell permeability or *in vitro* metabolism. A disadvantage is the possible inexistence of specific inhibitors, or the poor translation of *in vitro* enzymatic assay  $IC_{50}$  to the whole parasite assay  $IC_{50}$ .

In the present work, the BNIP derivatives were used in the attempt to chemically validate TcSir2rp1 as a potential drug target against Chagas disease. Some of the derivatives were active against the enzyme, and a significant portion was also active *in vitro* against amastigotes. One of the limitations of the study was the unavailability of a specific inhibitor of TcSir2rp1. BNIPSpd was the closest compound that could be considered as such, since it strongly inhibited the enzyme in a dose-dependent manner, and also had *in vitro* potency against amastigotes.

By contrast, the phenotypic strategy employed for *T. cruzi* was able to find a higher number of active inhibitors than the target-based approach, but also inhibitors with a high selectivity index for the parasite (section 4.2.1.1). It should be noted, however, that the phenotypic approach was started with a much higher number of starting compounds, and the chances of finding active hits were higher. Although the exact mechanism of action is not known for any of the compounds, this screening strategy presented some advantages: all hit compounds already possess adequate cell permeability, selectivity towards the parasite and have suitable *in vitro* drug metabolism and pharmacokinetics.

But which strategy should be prioritized? Recent data from the pharmaceutical industry as a whole indicate the trend. Analysis of the first in class molecular entities approved by the American Food and Drug Administration (FDA) in the years 1999 to 2008 reveal that 28 of those drugs were discovered by employing phenotypic assays in early drug discovery, whereas only 17 entities were discovered by target-based approaches [430].

The example of drug discovery for tuberculosis, an intracellular bacteria, is a clear example in infectious diseases drug discovery: despite the genome being sequenced in 1988 [431] and many well characterized targets having been reported since then, also with good targeting drugs, most of them have failed to advance as clinical leads [432]. By contrast, the phenotypic approach has led to the approval of drugs like bedaquiline [433] recently approved by the FDA [434], delamanid approved by the European Medicines Agency [435], or pretomanid [436] and Q203 [437] that have begun phase II and phase I clinical trials, respectively.

Finally, some lessons can be drawn from compounds that are used currently in the therapeutic for neglected tropical diseases and support the preference for phenotypic assays: first, they allow the exploitation of non-protein drug targets, like is the case of amphotericin B that acts through a mechanism involving the formation of pores at the ergosterol-rich membrane of *Leishmania* [438, 439]; second, they uncover pro-drugs, that are compounds that undergo bio-activation in parasite cells as are the examples of nitroheterocycles benznidazole and nifurtimox used in Chagas disease [352] or the case of allopurinol in leishmaniasis [440]; and finally, they would allow the identification of drugs like

arsenical compounds that are active because of selective concentration by trypanosomal transporters [441]. Any of this compounds and respective valid drug targets would be missed and could not be reliably predicted using current genomic tools extensively applied in target-based approaches.



# **PART VI**

## **Annexes**





### **6.1. Generation of molecular tools for essentiality evaluation of Sir2 related protein 1 from *Trypanosoma cruzi***

*Data not published*

#### **Objectives of the study:**

The genetic validation of *Trypanosoma cruzi* Sir2 related protein 1 (TcSir2rp1) is an important step in the validation of this protein as a drug target in the parasite. We describe the generation of the molecular tools necessary for the knockout of TcSir2rp1 by classic gene deletion by homology recombination, as well as the first two attempts at generating mutant *T. cruzi* cell lines.

#### **Main results:**

→ Two deletion constructs were successfully synthesized by fusion PCR, as well as an overexpression plasmid by traditional cloning;

→ In the attempts at generating mutants, although it was possible to obtain resistant cultures for some controls, no gene knockout parasites were obtained.

#### **Conclusion:**

Even though the synthesis of the molecular tools for the genetic manipulation of *T. cruzi* is relatively straightforward, the successful generation of mutant cell lines is difficult and time consuming. Additional attempts and the use of newer technologies should increase the chances of obtaining the desired genotype.

## METHODOLOGIES

### Parasite culture

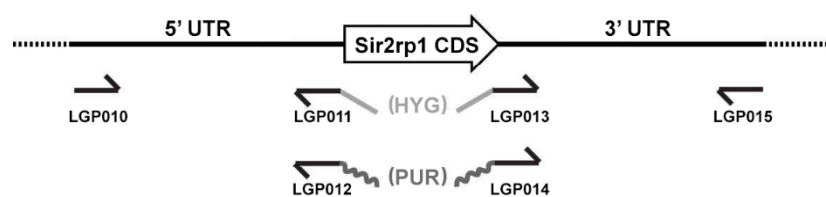
*Trypanosoma cruzi* epimastigotes from CL Brener or Y strain were grown at 28°C in LIT medium supplemented with 20 mg/L haemin and 10% heat inactivated FBS [442]. Cultures were maintained at logarithmic growth by dilution when density reached  $1 \times 10^7$  parasites/mL.

### Generation of knockout, overexpression and rescue constructs

The primers used for the generation of constructs for genetic manipulation were synthesized based on the CL Brener Esmeraldo-like sequence of *T. cruzi* Sir2 related protein 1 available in TriTrypDB as a template (Table 1, accession number: XP\_818420.1). All the constructs' synthesis accuracy was checked by DNA Sanger sequencing. Sir2rp1 gene replacement cassettes were generated by fusion PCR as previously described [443] (represented in Figure 1). Briefly, a region of DNA comprising the ~600 bp of the 5'-untranslated region (UTR) and the ~500 bp of the 3'-UTR amplified from genomic DNA (primers 10+11/12 and 13/14+15, respectively) were fused by PCR to the ORF of the selective markers for drug resistance genes PAC (puromycin N-acetyltransferase) and HYG (hygromycin phosphotransferase). The fusion constructs were posteriorly TA cloned into pGEM for amplification and cut from the backbone with NotI. The overexpression/rescue plasmid was generated by amplifying the coding sequence of TcSir2rp1 (Primer 22+23) from genomic DNA and cloned into pTREX with XbaI and XhoI restriction sites [444].

**Table 1.** Primers used in the present study.

Primers	Sequence
LGP010	TGACACATGCGTGCACTGAG
LGP011	GGTGAGTTCAGGCTTTTTCATTGGGAAGAAGTAATCCACCTGGC
LGP012	GTGGGCTTGTA CTGGTCATTGGGAAGAAGTAATCCACCT
LGP013	GTCCGAGGGCAAAGGAATAGGAGGAAAAGCGGCTGAAATAAAG
LGP014	GACCCGCAAGCCCGGTGCCTGAGAGGAAAAGCGGCTGAAATAAAG
LGP015	ATTTATCTTGCCTCTCCCCTCAC
LGP022	TCTAGAATGAATCAAGATAACGCCAACTTT
LGP023	CTCGAGTTATTTTCGGTCTGTCTGTGTGTA



**Figure 1.** Schematic of the fusion PCR protocol employed in the generation of knockout cassettes for TcSir2rp1.

### Parasites transfection and selection

Transfection of *T. cruzi* epimastigotes was performed with an Amaxa Nucleofector electroporator like described previously [422]. A total quantity of 5-10 µg of DNA was transfected into logarithmic growing parasites ( $1 \times 10^7$  per transfection) suspended in Human T-cell Nucleofector solution (100 µl). using the program U-33, V-33 or X-01. Twenty-four hours after transfection, selection drugs were added to the cultures of parasites: puromycin 2 µg/mL, geneticin 100 µg/mL and hygromycin 75-500 µg/mL.

## RESULTS AND DISCUSSION

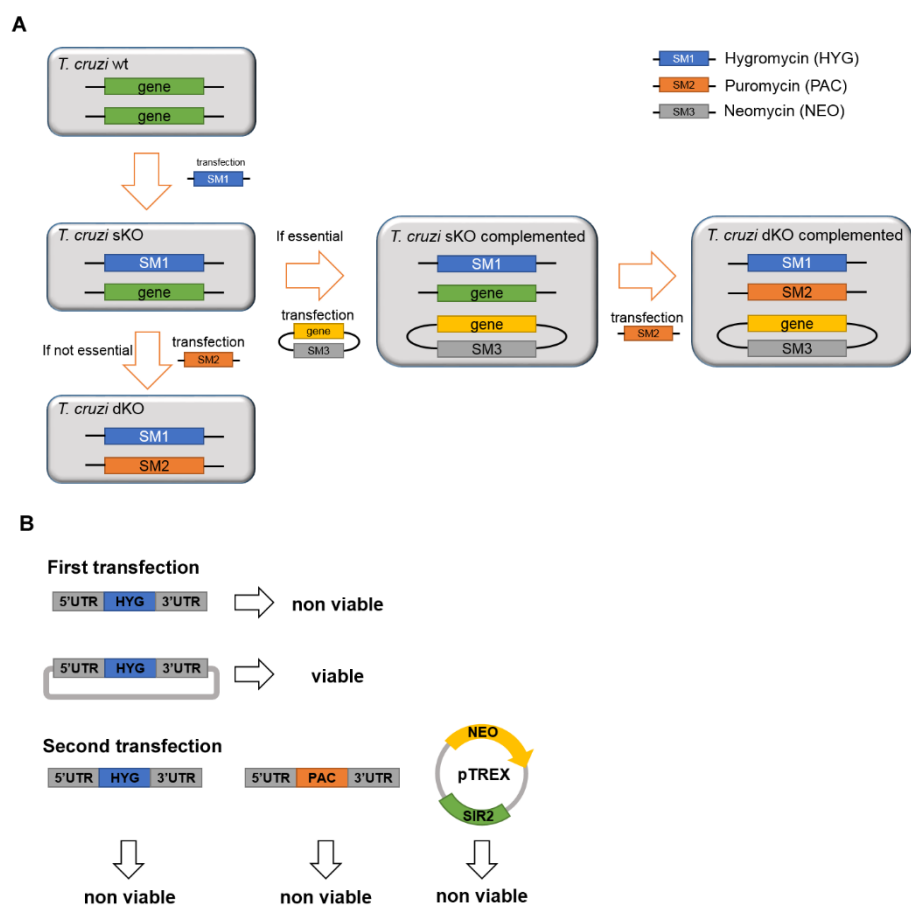
The evaluation of essentiality of a gene in *T. cruzi*, as well as in other pathogens, is an essential step in the characterization of the respective protein as a potential drug target. The lack of RNAi machinery has greatly hindered the evaluation of new drug targets in *T. cruzi*. Apparently some of its components have been lost or mutated during evolution [445, 446]. By comparison, in *T. brucei*, where RNAi is functional, the quantity of potential drug targets, as well as the overall understanding of its biology, is much more extensive. As a consequence, gene essentiality evaluation in *T. cruzi* has been relying in traditional homology recombination, and has not evolved with other gene editing technologies.

This work attempted the generation of mutant cell lines of *T. cruzi* where the gene of Sir2rp1 would be removed from the parasite genome. In order to achieve the deletion of the endogenous copies of the gene, two cassettes containing the PAC and HYG selection marker flanked by UTRs of TcSir2rp1 were synthesized (Figure 1). In addition to the deletion constructs, one plasmid for expression of an ectopic copy that could be used for overexpression or complementation for facilitated double endogenous allele replacement, was synthesized. The experimental approach is represented in Figure 2 A. Although the protocol in which the fusion PCR was based [443] reports a high efficiency in the double fusion (5'UTR + Selection Marker (SM) + 3'UTR), in this work it was only possible to obtain single fusions (5'UTR + (selection marker) SM and SM + 3'UTR) for both constructs. The final construct was then obtained by fusion of the two single fusions (5'UTR-SM + SM-3'UTR). Sequencing of the puromycin knockout cassette revealed no mutations in the selection marker, and only occasional indels in the UTRs. However, their frequency was considered to not interfere with homologous recombination. The cassette for hygromycin included one mutation that resulted in change of aminoacid in the coding sequence of the selective marker. However, when the construct was transfected in the circular pGEM vector (Figure 2 B), parasites were viable and successfully acquired resistance to hygromycin, suggesting that the mutation was not interfering with resistance to the drug.

In the first transfection experiment (Figure 2 B), 5 µg of HYG cassette both as a circular plasmid in pGEM and linearized by NotI was transfected using program U-33 and CL Brener strain epimastigotes. After 7 weeks of selection with hygromycin at 500 µg/mL, only the circular construct in pGEM-transfected parasites were viable, whereas the linearized construct did not produce any viable parasites up to 10 weeks of selection. The parasite is able to uptake circular DNA molecules and maintain them as extrachromosomal circular elements composed of head-to-tail tandem repeats of the vector [447], but is less amenable to gene deletion [422] which might explain the results obtained.

In the second transfection experiment (Figure 2 B), a higher quantity of each linearized DNA deletion cassette and circular pTREX plasmid was used (10 µg per transfection) in Y strain epimastigotes, using the V-33 program. Although by 4 weeks of selection, the negative control cultures (epimastigotes transfected without DNA and subject to selection drug) were dead, after 8 weeks of selection none of the DNA-transfected cultures had viable parasites, including the pTREX overexpression plasmid, indication a likely experimental or biologic limitation.

Additional attempts are recommended for the generation of TcSir2rp1 gene knockouts. CRISPR/Cas9 technology has recently been applied to *T. cruzi* genetics and should constitute an advancement over traditional techniques [58].



**Figure 2.** Transfections of *Trypanosoma cruzi*. **A)** Experimental approach for the generation of TcSir2rp1 null or facilitated null mutants. **B)** Transfection attempts with the knockout cassettes and overexpression/rescue plasmid.



# **PART VII**

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